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(54) Title: USE OF PRION CONVERSION MODULATING AGENTS

(57) Abstract: The use of Apolipoprotein B, Apoliporpotein E, fragments and mimetics thereof is provided for diagnostic, detection, prognostic and therapeutic applications in prion diseases. More specifically, the invention provides the use of Apolipoprotein B or fragments thereof for modulating or identifying modulators of the prion protein replication which are implicated in thepathogenesis of transmissible spongiform encephalopathics and other prion diseases.

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USE OF PRION CONVERSION MODULATING AGENTS

Field of the invention

This invention relates to the use of apolipoprotein B or apolipoprotein E or fragments or mimetics thereof for diagnostic, detection, prognostic and identifying modulators of the prion protein replication. More specifically, the invention provides the use of modulators of apolipoprotein B or fragments thereof for modulating the prion protein replication which are implicated in the pathogenesis of transmissible spongiform encephalopathies and other prion diseases.

Background of the invention

Creutzfeldt-Jakob disease (CJD) in humans and scrapie and bovine spongiform encephalopathy (BSE) in animals are some of the diseases that belong to the group of Transmissible Spongiform Encephalopathies (TSE), also known as prion diseases *(Prusiner, 1991)*. These diseases are characterized by an extremely long incubation period, followed by a brief and invariably fatal clinical disease (*Roos et al., 1973*). To date no therapy is available.

Although these diseases are relatively rare in humans, the risk for the transmissibility of BSE to humans through the food chain has seized the attention of the public health authorities and the scientific community *(Soto at al., 2001)*. Variant CJD (vCJD) is a new disease, which was first described in March 1996 *(Will et al., 1996)*. In contrast to typical cases of sporadic CJD (sCJD), this variant form affects young patients (average age 27 years old) and has a relatively long duration of illness (median 14 months vs. 4.5 months in traditional CJD). A link between vCJD and BSE was first hypothesized because of the association of these two TSEs in place and time *(Bruce, 2000)*. The most recent and powerful evidence comes from studies showing that the transmission characteristics of BSE and vCJD to mice are almost identical and strongly indicating that they are due to the same causative agent *(Bruce et al., 1997)*. Moreover, transgenic mice carrying a human or a bovine gene have now been shown to be susceptible to BSE and vCJD *(Scott et al., 1999)*. Furthermore, no other plausible hypothesis for the occurrence of vCJD has been proposed and intensive CJD surveillance in five European

countries, with a low exposure to the BSE agent, has failed to identify any additional cases. In conclusion, the most likely cause of vCJD is exposure to the BSE agent, probably due to dietary contamination with affected bovine central nervous system tissue.

The nature of the transmissible agent has been matter of passionate controversy. Further research, has indicated that the TSE agent differs significantly from viruses and other conventional agents in that it seems not to contain nucleic acids *(Prusiner, 1998)*. Additionally, the physicochemical procedures that inactivate most viruses, such as disrupting nucleic acids, have proved ineffective in decreasing the infectivity of the TSE pathogen. In contrast, the procedures that degrade protein have been found to inactivate the pathogen *(Prusiner, 1991)*. Accordingly, the theory that proposes that the transmissible agent is neither a virus nor other previously known infectious agent, but rather an unconventional agent consisting only of a protein recently gained widespread acceptability *(Prusiner, 1998)*. This new class of pathogen was called a "prion", short for "proteinaceous infectious particle". In TSE, prions are composed mainly of a misfolded protein named PrP^{Sc} (for scrapie PrP), which is a post-translationally modified version of a normal protein, termed PrP^{C} *(Cohen et al., 1998)*. Chemical differences have not been detected to distinguish these two PrP isoforms and the conversion seems to involve a conformational change whereby the α-helical content of the normal protein diminishes and the amount of β-sheet increases *(Pan et al., 1993)*. The structural changes are followed by alterations in the biochemical properties: PrP^{C} is soluble in non-denaturing detergents, PrP^{Sc} is insoluble; PrP^{C} is readily digested by proteases (also called protease sensitive prion protein) while PrP^{Sc} is partially resistant, resulting in the formation of a N-terminally truncated fragment known as PrPres (protease resistant prion protein) *(Cohen et al., 1998)*.

The notion that endogenous PrP^{C} is involved in the development of infection is supported by experiments in which endogenous PrP gene was knocked out where the animals were both resistant to prion disease and unable to generate new infectious particles *(Bueler et al., 1993)*. In addition, it is clear that during the time between the inoculation with the infectious protein and the appearance of the clinical symptoms, there is a dramatic increase in the amount of PrP^{Sc}.

These findings suggest that endogenous PrP^C is converted to PrP^{Sc} conformation by the action of an infectious form of the PrP molecule *(Soto et al., 2001)*. Prion replication is hypothesized to occur when PrP^{Sc} in the infecting inoculum interacts specifically with host PrP^C, catalyzing its conversion to the pathogenic form of the protein. A physical association between the two isoforms during the infectious process is suggested by the primary sequence specificity in prion transmission *(Telling et al., 1994)* and by the reported *in vitro* generation of PrP^{Sc}-like molecules by mixing purified PrP^C with PrP^{Sc} *(Saborio et al., 2001)*. However, the exact mechanism underlying the conversion is not known.

Investigations with chimeric transgenes showed that PrP^C and PrP^{Sc} are likely to interact within a central domain delimited by codons 96 and 169 *(Prusiner, 1996)* and synthetic PrP peptides spanning the region 109-141 proved to be able to bind to PrP^C and compete with PrP^{Sc} interaction *(Chabry et al., 1998)*.

Based on data with transgenic animals, it has been proposed that additional brain factors present in the host are essential for prion propagation *(Telling et al., 1995)*. It has been demonstrated previously that prion conversion does not occur under experimental conditions where purified PrP^C and PrP^{Sc} are mixed and incubated *(Saborio et al., 1999)* but that the conversion activity is recovered when the bulk of cellular proteins are added back to the sample *(Saborio et al., 1999)*. This finding provides direct evidence that other factors present in the brain are essential to catalyse prion propagation.

The observation that cholesterol depletion decreases the formation of PrP^{Sc} whereas sphingolipid depletion increases PrP^{Sc} formation, suggested that "lipid rafts" (lipid domains in membranes that contain sphingolipids and cholesterol) may be the site of the PrP^c to PrP^{Sc} conversion reaction involving either a raft-associated protein or selected raft lipids *(Fantini et al., 2002)*. However, the role of lipid rafts in prion infectivity is still unclear.

Several *in vitro* methods of detections of prions in a sample have been developed. The set of known detection methods, include PrP^{Sc} detection methods using specific ligand carriers selected from aminoglycans, fibronectin and Apolipoprotein A (*WO*

02/065133); methods using the monoclonal antibodies selected from Gö138, 3B5 and 12F10 (*Schulz et al., 2000*); methods based on the formation of a complex between PrP^{Sc} and Apolipoprotein H (*WO 03/005037*); or methods based on the PrP^{Sc} *in vitro* amplification called protein misfolding cyclic amplification (PMCA) described in *Saborio et al., 2001* and *Lucassen et al., 2003.*

Apolipoprotein B is the major protein component of the two known atherogenic lipoproteins, Low Density Lipoproteins (LDL) and remnants of triglyceride-rich lipoproteins. The apolipropotein B concentration is considered to be a direct reflection of the number of atherogenic particles in the blood and has been proposed as a parameter for determining the risk of atherosclerosis.

Apolipoprotein E is a constituent of several plasma lipoprotein such as chylomicrons, very low-density lipoproteins (VLDL), and high-density lipoproteins (HDL) (*Lehninger et al., 1993*).
Apolipoprotein E has recently emerged as a major genetic risk factor for Alzheimer's disease, a neurodegenerative disorder (*US 6,022,683*) and upregulated in the cerebrospinal fluid of patients with variant CJD and Alzheimer's disease compared to patients with sporadic CJD (*Choe et al., 2002*). The Apolipoprotein E 4/4 phenotype is associated with increased risk of coronary heart diseases and Creutzfeld-Jakob disease (*Golaz et al., 1995*). Apolipoprotein E gene expression was found to be increased in astrocytes associated with the neuropatholigical lesions in a scrapie animal model (*Dietrich et al., 1991*).
Apolipoprotein E was found to recognise a shared structural motif of amyloids and prion which, after induction, can accelerate the adoption of a beta-sheet conformation (*Baumann et al., 2000*).

Apolipoprotein B and E are ligands for the LDL receptor and are known for its prominent role in cholesterol transport and plasma lipoprotein metabolism via LDL receptor interactions *(Segrest et al., 2001; Clavey et al., 1991).*

One approach to the treatment and prevention of prion diseases has been to develop agents for blocking the transformation of PrP^{c} into PrP^{Sc}. Some agents proposed were Congo red dye (*US 5,276,059*), nerve growth peptides (*US 5,134,121*), fragments of prion proteins (*US 6,355,610*), compounds that reduces Apolipoprotein E release in the brain tissue (*US 2002/0155426*), therapeutic agents that prevent Apolipoprotein E4 to interact with neuronal LDL receptor-related protein (*WO 97/14437*), compounds that increase Apolipoprotein E levels (*WO 99/15159*) and beta-sheet breaker peptides (*US 5,948,763*).

It would be desirable to develop new methods for identifying and inhibiting the prion conversion factor(s).

Summary of the invention

It is an object of the invention to provide a use of peptides or proteins in an assay for the detection of PrP^{Sc} formation in a sample.

It is also an object of the invention to provide a use of peptides or proteins in a screening assay for identifying compounds that modulate the conversion of PrP^{c} into PrP^{Sc}.

It is further an object of the invention to provide a substance which is suitable for the treatment of, and/or prevention of, and/or delaying the progression of prion related disorders, notably, bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD).

In a first aspect, the invention provides a use of a peptide or a protein selected from Apolipoprotein B; a fragment or mimetic thereof; Apolipoprotein E and a fragment or mimetic thereof, in an assay for the detection of PrP^{Sc} formation in a sample.

In a second aspect, the invention provides a use of a peptide or a protein selected from Apolipoprotein B; a fragment or mimetic thereof; Apolipoprotein E and a fragment or mimetic thereof, in a screening assay for identifying compounds that modulate the conversion of PrP^{c} into PrP^{Sc}.

In a third aspect, the invention provides a use of a modulator, preferably an inhibitor or an antagonist, of a peptide or a protein, wherein the peptide or the protein is selected from Apolipoprotein B; a fragment and a mimetic thereof, for the preparation of a pharmaceutical composition for the treatment of a prion disease, notably, bovine spongiform encephalopathy (BSE) and a Creutzfeld-Jacob Disease (CJD).

In a fourth aspect, the invention provides a method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B; a fragment or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof; (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

In a fifth aspect, the invention provides a method of determining a marker that predisposes a subject to a prion disease, comprising (i) measuring a level of a protein selected from Apolipoprotein B and a fragment thereof; and (ii) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease.

In a sixth aspect, the invention provides a method for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

In a seventh aspect, the invention provides a method for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof (a) in the presence of said compound and (b) in the

absence of said compound; (ii) contacting the sample obtained from step (i) a and (i) b with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound.

In a eighth aspect, the invention provides an assay for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

In a ninth aspect, the invention provides a screening assay for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof (a) in the presence of said compound and (b) in the absence of said compound; (ii) contacting the sample obtained from step (i) a and (i) b with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound.

Detailed description of the invention

The following paragraphs provide definitions of various terms, and are intended to apply uniformly throughout the specification and claims unless an otherwise expressly set out definition provides a different definition.

The term "Gerstmann-Strassler-Scheinker Disease" abbreviated as "GSS" refers to a form of inherited human prion disease. The disease occurs from an autosomal dominant disorder. Family members who inherit the mutant gene succumb to GSS.

The term "prion" shall mean a transmissible particle known to cause a group of such transmissible conformational diseases (spongiform encephalopathies) in humans and animals. The term "prion" is a contraction of the words "protein" and "infection" and the particles are comprised largely if not exclusively of PrP^{Sc} molecules.

"Prions" are distinct from bacteria, viruses and viroids. Known prions include those which infect animals to cause scrapie, a transmissible, degenerative disease of the nervous system of sheep and goats as well as bovine spongiform encephalopathies (BSE) or mad cow disease and feline spongiform encephalopathies of cats. Four prion diseases known to affect humans are Kuru, Creutzfeldt-Jakob Disease (CJD), Gerstmann-Strassler-Scheinker Disease (GSS), and fatal familial insomnia (FFI) *(Prusinier, 1991)*. As used herein prion includes all forms of prions causing all or any of these diseases or others in any animals used -- and in particular in humans and in domestic farm animals.

The term "lipid rafts" refers to small platforms, composed of sphingolipids and cholesterol in the outer exoplasmic layer, connected to Cholesterol in the inner cytoplasmic layer of the bilayer that have been reviewed recently *(Simons et al., 2000)*. Lipid rafts can be isolated as they are insoluble in certain detergents such as triton X-100 at 4°C. Therefore, rafts can be purified as detergent-insoluble membranes (DIMs) or detergent-resistant membranes (DRMs) by ultracentrifugation on sucrose gradients. Rafts are enriched in GPI-anchored proteins, as well as proteins involved in signal transduction and intracellular trafficking. In neurons, lipid rafts act as platforms for the signal transduction initiated by several classes of neurotrophic factors *(Tsui-Pierchala et al., 2002)*. Example for lipid rafts extraction is given in Example n° 2 §c.

The term "prion conversion factor" refers to a factor comprising proteins, lipids, enzymes or receptors that acts as a co-factor or auxiliary factor involved in the process of conversion of PrP^{C} into PrP^{Sc} and favors the onset and/or progression of the prion disease.

The terms "standardized prion preparation", "prion preparation" and the like are used interchangeably herein to describe a composition containing prions which composition is obtained for example from brain tissue of mammals substantially the same genetic material as relates to PrP proteins, e.g. brain tissue from a set of mammals which exhibit signs or prion disease or for example a composition which is obtained from chronically prion infected cells.

The terms "sensitive to infection", "sensitive to prion infection" and the like are use for a material from a mammal, including cells, that can be infected with an amount and type of prion which would be expected to cause prion disease or symptoms.

By analogy, the terms "resistant to infection", "resistant to prion infection" and the like are used for a material from a mammal, including cells which has the characteristic to be resistant when infected with an amount and type of prion which would be expected to cause prion disease or symptoms and remain uninfected even after several infective prion material inoculations.

The term "sample" refers to a biological extract from a mammal, including cell sample, body fluid, genetic material such as brain homogenate, cells, lipid rafts or purified peptides and proteins.

The term "incubation time" shall mean the time from inoculation of an animal with a prion until the time when the animal first develops detectable symptoms of disease resulting from infection, it also means the time from inoculation of material from a mammal, e.g. brain homogenate, cells, lipid rafts from cells, with prion until the time when the prion infection is detectable such as through the conversion of PrP^{C} into PrP^{Sc}. Several methods of detection of prion infection and PrP conversion are known by a person skilled in the art.

The terms "fraction" or "fragment" refer to any fragment of the polypeptidic chain of the compound itself, alone or in combination with related molecules or residues bound to it, for example residues of sugars or phosphates, or aggregates of the original

polypeptide or peptide. Such molecules can result also from other modifications which do not normally alter primary sequence, for example *in vivo* or *in vitro* chemical derivativization of peptides (acetylation or carboxylation), those made by modifying the pattern of phosphorylation (introduction of phosphotyrosine, phosphoserine, or phosphothreonine residues) or glycosylation (by exposing the peptide to enzymes which affect glycosylation e.g., mammalian glycosylating or deglycosylating enzymes) of a peptide during its synthesis and processing or in further processing steps.

The terms "modulator" or "modulatory compound" refer to molecules that modify the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, metabolism) of the natural protein. "Modulators" or "modulatory compounds" include "agonists" and antagonists". Modulators" include peptides, proteins or fragments thereof, peptidomimetics, organic compounds and antibodies.

The term "mimetic" refer to molecules that mimic the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, metabolism) of a natural protein. These compounds have for example the property to either enhance a property of the natural protein (i.e. to lead to the same activity when the compound is added to the natural protein as obtained with an increase in concentration in the natural protein) or to exhibit the same property as a natural protein (i.e. to lead to the same activity when the compound replaces the natural protein). "Mimetics" include peptides, proteins or fragments thereof, peptidomimetics and organic compounds. Examples of Apolipoprotein E mimetics are described in *US 20020128175* and *WO 2004043403*.

The terms "inhibitor" or "antagonist" refer to molecules that alter partially or impair the functions and/or properties (such as receptor binding, lipid affinity, enzyme interaction, structural arrangement, synthesis, secretion, metabolism) of the natural protein. "Inhibitors" or "antagonists" include peptides, proteins or fragments thereof, peptidomimetics, organic compounds and antibodies. Examples of Apolipoprotein B antibodies are described in *Choi et al., 1997* and in *Wang et al., 2000*. Examples of

Apolipoprotein antagonists can be antagonists that alter or impair the role of Apolipoproteins B or E in the cholesterol transport pathway. Examples of compounds that alter Apolipoprotein B secretion or synthesis are described in *US 6,369,075*, *US 6,197,972*, *WO 03/002533* and *WO 03/045921*. Other "modulators" or "antagonists" can be modulators of the LDL receptor, preferably LDL-receptor antagonists such as anti-LDL receptor antibodies. Examples of monoclonal antibodies to the LDL receptor are given in *WO 01/68710*.

The term "protein misfolding cyclic amplification assay" or "PMCA assay" is an assay that for the diagnosis or detection of conformational diseases which comprises a cyclic amplification system to increase the levels of the pathogenic conformer such as described for example in *WO 02/04954*.

The term "marker" for a disease refers to a biological parameter or value including a genetic character, inherited protein mutation(s), blood level of a protein or an enzyme that is different from the average value in a heterogeneous population of individuals and whose occurrence correlates with the occurrence of said disease with a statistical significance. A "marker" for a disease or condition is typically defined as a certain cut-off level of a said biological variable. A "marker" provides basis for determining the risk (probability of occurrence) of a disease in a subject.

The term "complex" includes the formation of an entity by the interaction of several molecules, several proteins, several peptides together or with a receptor. These interactions may be reversible and/or transient. These interactions may induce changes in the properties of the interacting molecules, proteins, peptides or receptors.

By "effective amount", it is meant a concentration of peptide(s) that is capable of slowing down or inhibiting the formation of PrP^{Sc} deposits, or of dissolving preformed deposits. Such concentrations can be routinely determined by those of skill in the art. It will also be appreciated by those of skill in the art that the dosage may be dependent on the stability of the administered peptide. A less stable peptide may require administration in multiple doses.

The preparation of antibodies is known by the person skilled in the art. It is referred by "antibody" to a monoclonal antibody, chimeric antibody, humanized antibody, anti-anti-Id antibody or fragment thereof which specifically recognises and binds to Apo B or Apo E and fragments thereof. For example, monoclonal antibodies are obtained though the generation of hybridoma cells lines producing monoclonal antibodies capable of specifically recognising and binding Apo B and/or fragments thereof. More specifically, these monoclonal antibodies are capable of specifically recognising and binding Apo B. A monoclonal antibody can be prepared in a conventional manner, e.g. by growing a cloned hybridoma comprising a spleen cell from a mammal immunized with hApo B and a homogenic or heterogenic lymphoid cell in liquid medium or mammalian abdomen to allow the hybridoma to produce and accumulate the monoclonal antibody. Preferably, the antibody specifically recognises and binds to Apo B-LDL recognizing fragments.

The present invention provides compounds capable of controlling, including increasing and/or inhibiting, the conversion of PrP^{C} into PrP^{Sc} in prion diseases.

The activity of the compounds of the invention in controlling the conversion of PrP^{C} into PrP^{Sc} in prion diseases can be detected using, for example, an *in vitro* assay, such as that described by *Saborio et al., 2001* which measures the ability of compounds of the invention to modulate the conversion of PrP^{C} into PrP^{Sc}. Results are reported in the Examples.

In one embodiment, the invention provides a use of a peptide or a protein selected from Apolipoprotein B; a fragment thereof or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof, preferably Apolipoprotein B; a fragment thereof and a mimetic thereof; in an assay for the detection of PrP^{Sc} formation in a sample.

In one further embodiment of the invention, the peptide or the protein selected from Apolipoprotein B; a fragment thereof or a mimetic thereof; Apolipoprotein E; a

fragment thereof and a mimetic thereof, preferably Apolipoprotein B or a fragment thereof; used in an assay for the detection of PrP^{Sc} formation in a sample binds and/or forms a complex with the LDL receptor.

In another embodiment, the invention provides a use of a peptide or a protein selected from Apolipoprotein B; a fragment thereof or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof, preferably Apolipoprotein B or a fragment thereof, in a screening assay for the identifying compounds that modulate the conversion of PrP^{c} into PrP^{Sc}.

In another further embodiment of the invention, the peptide or the protein selected from Apolipoprotein B; a fragment thereof or a mimetic; Apolipoprotein E; a fragment thereof and a mimetic thereof, preferably Apolipoprotein B or a fragment thereof, thereof, is used in a screening assay for the identifying compounds that modulate the conversion of PrP^{c} into PrP^{Sc} binds and/or forms a complex with the LDL receptor.

In a further embodiment of the invention, the assay is a Protein Misfolding Cyclic (PMCA) assay.

In a preferred embodiment of the invention, the Protein Misfolding Cyclic (PMCA) assay uses normal brain homogenate as a source of normal PrP^{c} and prion conversion factor.

In a further embodiment of the invention, the protein according to the invention is Apolipoprotein B.

In a preferred embodiment of the invention, the Protein Misfolding Cyclic (PMCA) assay uses cell lysates or lipid rafts extracted from prion infection sensitive neuroblasma cells, such as line N2a, described in Example 2, and equivalent, as a source of normal PrP^{c} and prion conversion factor. Lipid raft fractions can also be purified directly from the brain to serve as a source of substrate for PMCA.

In a preferred embodiment, the invention provides a use of Apolipoprotein Bin an assays for the detection of PrP^C in a sample, wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using lipid rafts from infection sensitive neuroblasma cell line N2a as a source of normal PrP^C and substrate.

In another embodiment, the invention provides a use of a modulator, preferably an inhibitor or an antagonist, of a peptide or a protein, wherein the peptide or the protein is selected from Apolipoprotein B; a fragment thereof or a mimetic thereof for the preparation of a pharmaceutical composition for the treatment of a prion disease, notably, bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD). The modulator modifies for example the functions and/or properties of Apolipoliprotein B or of a fragment thereof.

In a further embodiment of the invention, the modulator, preferably an inhibitor or an antagonist, of a peptide or a protein, wherein the peptide or the protein is selected from Apolipoprotein B; a fragment thereof and a mimetic thereof which modifies, preferably inhibits the binding and/or the formation of a complex between Apolipoprotein B and the LDL receptor. An example of such modulator can be a LDL receptor modulator, such as a LDL-receptor antagonist such as an anti-LDL receptor antibody.

In a preferred embodiment of the invention, the modulator is an antagonist to Apolipoprotein B or a fragment thereof.

In a further preferred embodiment of the invention, the modulator is an antibody raised against Apolipoprotein B or against a fragment thereof.

In another preferred embodiment of the invention, the modulator is an antibody raised against Apolipoprotein B.

In another preferred embodiment of the invention, the modulator is an antibody raised against a fragment of Apolipoprotein B, which fragment is of, or about, a molecular weight selected from 30, 35 and 40 kDa.

In another preferred embodiment of the invention, the modulator is an antibody raised against a fragment of Apolipoprotein B, which fragment comprises a sequence selected from fragments taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

In a preferred embodiment of the invention, the peptide or protein is selected from Apolipoprotein B or a fragment thereof.

In a preferred embodiment of the invention, the peptide or protein contains the sequence of SEQ ID NO: 3.

In another preferred embodiment of the invention, the peptide or protein is a fragment which is of, or about, a molecular weight selected from 30, 35 and 40 kDa.

In another preferred embodiment of the invention, the peptide or protein is a fragment of Apolipoprotein B, comprising a sequence selected from fragments, taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

In an embodiment of the invention, the invention provides a method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B; a fragment or a mimetic thereof; Apolipoprotein E; a fragment thereof and a mimetic thereof; preferably Apolipoprotein B or a fragment thereof, (ii) contacting the sample obtained from step (i) with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample. The sample from the subject includes a biological extract from a mammal such as cell sample, genetic material, body fluid, brain homogenate, cells and lipid rafts.

In another embodiment of the invention, the invention provides a method of determining a marker that predisposes a subject to a prion disease, comprising (i)

measuring a level of a protein selected from Apolipoprotein B and a fragment thereof in said sample; (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease. The maker includes a biological parameter or value such as a genetic character, inherited protein mutation(s), blood level of a protein or an enzyme.

In another embodiment of the invention, the invention provides a method for the detection of PrP^{Sc} formation within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof or a mimetic thereof; Apolipoprotein E, a fragment thereof and a mimetic thereof, preferably Apolipoprotein B or a fragment thereof (ii) contacting the sample obtained from step (i) with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample. The sample can be a biological preparation for which the presence of prion is to be detected for quality control reasons and/or a sample extracted from a subject that is suspected of suffering of such a disease, including a biological extract from a mammal such as cell sample, genetic material, body fluid, brain homogenate, cells and lipid rafts.

In another embodiment of the invention, the invention provides a method for identifying, in a sample, a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^C into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof or a mimetic thereof; Apolipoprotein E, a fragment thereof and a mimetic thereof, preferably Apolipoprotein B or a fragment thereof (ii) contacting the sample obtained from step (i) (a) in the presence of said compound and (b) in the absence of said compound; (iii) contacting the sample obtained from step (i) a and (i) b, with PrP^C or PrP^C containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said compound. The modulator, includes antibodies, inhibitors of

Apolipoproteins B binding, including binding to the LDL receptor, and/or secretion and/or synthesis.

Still another embodiment of the present invention, is a method for treating or preventing a prion disease such as bovine spongiform encephalopathy (BSE) and Creutzfeld-Jacob Disease (CJD), wherein the method comprises administering an effective dose of the above-mentioned modulator of a peptide or a protein, wherein the peptide or the protein is selected from Apolipoprotein B and a fragment thereof, to a subject in the need thereof, wherein the subject can be human or animal.

In a preferred method of use of the modulators, preferably inhibitors, administration of the modulators is by injection or infusion, at periodic intervals. The administration of a compound of the invention may begin before any symptoms are detected in the patient, and should continue thereafter.

The above-mentioned modulatory compounds of the present invention may be administered by any means that achieves the intended purpose. For example, administration may be by a number of different routes including, but not limited to subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intra-cerebral, intrathecal, intranasal, oral, rectal, transdermal, intranasal or buccal. Preferably the compounds of the invention are administered by subcutaneous, intramuscular or intravenous injection or infusion.

Parenteral administration can be by bolus injection or by gradual perfusion over time. A typical regimen for preventing, suppressing, or treating prion related disorders, comprises either (1) administration of an effective amount in one or two doses of a high concentration of modulatory in the range of 0.5 to 10 mg of peptide, more preferably 0.5 to 10 mg of peptide, or (2) administration of an effective amount of the peptide in multiple doses of lower concentrations of modulatory compounds in the range of 10-1000 µg, more preferably 50-500 µg over a period of time up to and including several months to several years. It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, concurrent treatment, if any,

frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients which are known in the art. Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspension of the active compound as appropriate oily injections suspensions may be administered.

In another embodiment of the invention is provided an assay for the detection of the formation of PrP^{Sc} within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof or a mimetic thereof; Apolipoprotein E, a fragment thereof and a mimetic thereof, preferably Apolipoprotein B or a fragment thereof (iii) contacting the sample obtained from step (iii) contacting the sample obtained from step (ii) with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iv) determining the presence and/or amount of PrP^{Sc} in said sample. The sample can be a biological preparation for which the presence of prion is to be detected for quality control reasons and/or a sample extracted from a subject that is suspected of suffering of such a disease, including a biological extract from a mammal such as cell sample, genetic material, body fluid, including blood, serum, plasma, brain homogenate, cells and lipid rafts.

In another embodiment of the invention, is provided a screening assay for identifying a compound which modulates, preferably inhibits or antagonizes, the transition of PrP^{C} into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof or a mimetic thereof; Apolipoprotein E; a fragment thereof or a mimetic thereof, preferably Apolipoprotein B or a fragment thereof (a) in the presence of said compound and (b) in the absence of said modulatory compound; (ii) contacting the sample obtained from step (i) a and (i) b with PrP^{C} or PrP^{C} containing mixtures, such as brain homogenates, cell lysates, lipid rafts preparation; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said

compound and (b) in the absence of said modulatory compound. The modulator, includes antibodies, inhibitors of Apolipoproteins B and/or secretion and/or synthesis.

In further embodiment of the invention, is provided a diagnostic kit for use in the assay of the invention, comprising a probe for receiving a sample and a peptide or a protein selected from Apolipoprotein B; a fragment thereof and a mimetic thereof; Apolipoprotein E, a fragment thereof and a mimetic thereof. The kit of the invention comprises kits having multi-well microtitre plate and/or multi-well sonicator.

In a still further embodiment of the invention, is provided an apparatus for use in the methods of the invention or in the assays of the invention. The apparatus of the invention comprises apparatus that have a microtitre plate and/or multi-well sonicator.

In a preferred embodiment, the prion disease is bovine spongiform encephalopathy (BSE).

In a preferred embodiment, the prion disease is sporadic, variant, familial or iatrogenic Creutzfeld-Jacob Disease (CJD).

The present invention has been described with reference to the specific embodiments, but the content of the description comprises all modifications and substitutions, which can be brought by a person skilled in the art without extending beyond the meaning and purpose of the claims.

The invention will now be described by means of the following Examples, which should not be construed as in any way limiting the present invention. The Examples will refer to the Figures specified here below.

Brief description of the drawings:

Figure 1 shows *in vitro* prion replication on Hamster brain homogenate by PMCA assay in presence and absence of a cholesterol-depleting agent (Example 1§b). Samples contain 5% normal hamster brain homogenate incubated for 30 min at 4°C

with 0, 5, 10 or 20 mM (final concentration) of methyl-β-cyclodextrin (MβCD). Aliquots of scrapie brain homogenate are added to reach a 3200- (top panel) and 12800-fold (bottom panel) dilution. Half of the samples are frozen immediately as a control without amplification (PMCA "–") and the other half are subjected to 10 cycles of PMCA (PMCA "+"). Prion replication is evaluated by Western Blot after treatment of the samples with PK (100µg/ml for 60 min). The first lanes in each blot corresponds to the normal brain homogenate not treated with PK.

Figure 2 shows the effect of Apolipoproteins B, E and J *in vitro* prion replication on Hamster brain homogenate by PMCA assay (Example 1§c). Samples containing 5% normal hamster brain homogenate are incubated with different quantities of human Apolipoprotein B (2A), human Apolipoprotein E (2B) or murine Apolipoprotein J (2C) for 30 min at 4°C. Aliquots of scrapie brain homogenate are added to reach a 3200- (left panel) or 12800-fold (right panel) dilution. Half of the samples are frozen immediately as a control without amplification (PMCA "–") and the other half are subjected to 10 cycles of PMCA (PMCA "+"). Prion replication is evaluated by western blot after treatment of the samples with PK (100µg/ml for 60 min). The first lanes in each blot corresponds to the normal brain homogenate not treated with PK.

Figure 3 reports differential sensitivity of N2a sub-clones to infection by Scrapie revealed by exposure to anti-PrP 6H4 mabs (Example n° 2 §b). Proteinase K (PK) exposure shows were the PrP^{Sc} isoform (Proteinase K resistant) is present. The two sub-clones highlighted #23 and #60 are chosen respectively as representatives of prion infection resistant and sensitive cells. 'N2a' shows uninfected N2a cells processed in parallel. Controls for blotting and PK digestion show 1µl normal or scrapie brain extract diluted in 80µl lysis buffer and processed in parallel.

Figure 4 shows the characterization of PrP in lipid rafts from sub-clones prion infection resistant (#23) and sensitive (#60) N2a (Example 2 §c). **Figure 4A** shows PrP quantification by Western blotting in lipid rafts which are extracted from prion infection resistant (#23) and sensitive (#60) cells. The distribution of PrP in the total

extract (25µg loaded) (1), the sucrose sample layer after centrifigation (25µg loaded) (2) and the bouyant lipid raft fraction (4µg loaded) (3) are presented. **Figure 4B** shows PrP content and glycosylation pattern of the two sub-clones #23 and #60 by Western blotting with anti-PrP. Three independent preparations of lipid rafts prion infection resistant (#23) and sensitive (#60) cells were analysed. Equal amounts (4µg) of rafts proteins were analysed in each case. **Figure 4C** shows the same membrane after stripping and re-probing with anti-actin which confirms the similarity in protein loading.

Figure 5 presents the *in vitro* conversion activity of lipid rafts from sub-clones prion infection resistant (#23) and sensitive (#60) N2a using PMCA (Example 2 §d). *Upper panel:* Lipid rafts are isolated from prion infection resistant (#23) and sensitive (#60) cells. Preparations are mixed in a ratio 100:1 with 10% RML brain homogenate and aliquots are frozen immediately, incubated for 15h at 37 °C or subjected to 15 cycles of PMCA. Lanes 1: initial mixture without PK digestion; lanes 2: initial mixture digested 10ug/ml PK 1hr 37 C; lanes 3: mixture incubated 37 °C PK digested as in lane 2; lanes 4: 15 cycles of PMCA followed by PK digestion as in lane 2. Lane 5 shows the migration and cross-reactivity with anti-PrP of PK alone. *Lower panel:* Following western blotting the membrane is stained with Coomasie blue to confirm that digestion with PK was complete.

Figure 6 shows the inhibitory effect on Prion replication in prion infection sensitive cells induced by Anti-hApoB polyclonal antibody (Example 2 §e).

Chronically infected #60 sensitive cells were cultured in 24 well culture dishes in the presence of increasing amounts (0-2mg/ml) of a goat polyclonal antibody against human ApoB (Chemicon) or against a corresponding series of naïve goat IgG. The level of PrP replication was determined by quantitative dot blotting and expressed as chemiluminescent intensity/mg protein. In the graph, for each antibody concentration the chemiluminescent intensity is expressed as a percentage of the value obtained without the antibody. Higher concentrations of anti-hApoB antibody have an inhibitory effect on PrP replication.

Figure 7 shows 2D separations of lipid raft proteins from N2a cells (Example 3). Lipid rafts are isolated from prion infection sensitive cells (#60) and 2 aliquots of 25 µg are precipitated with acetone and processed for 2D analysis min the 1st dimension spanning pH ranges 3-10 (7A) or 6-11 (7B). After SDS-PAGE separation in the second dimension, gels are stained using the silver express kit (Invitrogen). Arrow indicates the same protein on both gels (7A and 7B). Proteins within the rectangle shown in B are compared between lipid raft from the prion infection sensitive sub-clone #60, (C) and resistant subclone #23, (D). Arrows indicate proteins which are more abundant in resistant cells.

Abbreviations:

Apo B (Apolipoprotein B; Apo E (apolipoprotein E); Apo J (Apolipoprotein J); BCA (Bicinchoninic Acid); CHAPS (3-((3-cholamidopropyl)dimethylammonio)-1-propanesulfonate); CNS (central nervous system); BSE (bovine spongiform encephalopathy); CJD (Creutzfeldt-Jakob Disease); DiI (1,1-dioctadecyl-3,3,3 ,3 – tetramethylindocarbocyanine perchlorate); DIM (Detergent-Insoluble Membrane); DMEM (Dulbecco's Modified Eagle Medium); DRM (Detergent-Resistant Membrane); DTT (1,4-Dithio-D,L-threitol); IPG (Immobilized PH Gradient); IEF (Isoelectric Focusing); FCS (Fetal Calf Serum); FFI (Fatal Familial Insomnia); GSS (Gerstmann-Strassler-Scheinker Disease); hr (hour); HRP (Horseradish Peroxidase); kDa (KiloDalton); LDL (Low Density Lipoprotein); µg (microgram); µl (microliter); min (minute); MßCD (methyl-β-cyclodextrin); mM (millimolar); MS (mass spectrometry); PBS (Phosphate Buffered Sulfate); PK (proteinase K); PMCA (Protein Misfolding Cyclic Amplification); PMSF (Phenylmethanesulfonyl Fluoride); PrP (prion protein); PrP^{C} (normal, non-pathogenic conformer of PrP); PrP^{Sc} (pathogenic or "scrapie" isoform of PrP which is also the marker for prion diseases); PVDF (polyvinylidene difluoride); RPM (Rotation per minute); RML (Rocky Mountain Laboratory); RT-PCR (reverse transcriptase polymerase chain reaction); SDS (Sodium Dodecyl Sulfate); V (Volt); Vol. (volume).

EXAMPLES

The invention will be illustrated by means of the following examples which are not to be construed as limiting the scope of the invention.

The following examples illustrate preferred compounds and methods for determining their biological activities.

PrP scrapie used as infection innoculum is RML (Rocky Mountain Laboratory) strain.

Anti-PrP 6H4 monoclonal antibodies were purchased from Prionics.

Proteinase K was obtained from Boerhinger Ingelheim and Methyl.β.cyclodextrin from Sigma.

Purified and delipidated human Apolipoprotein B (Apo B) and Apolipoprotein E (Apo E) were obtained from Chemicon.

Anti-apo B and anti-apo E are goat polyclonal antibodies against human Apo B and human Apo E, respectively obtained from Chemicon and dialysed against PBS to eliminate sodium azide.

Total goat IgG was purchased from Pierce and dialyzed against PBS.

Mouse neuroblasma N2a cell line was obtained from ATCC.

Murine Apo J (Apo J) was obtained in-house as described in PCT/EP2004/05037.

DiI labeled LDL was obtained from Molecular Probes (L-3482).

EXAMPLE 1: *In vitro* prion replication in brain homogenate through PMCA assay:

The influence of cholesterol and some of the apolipoproproteins on prion replication in vitro is analysed through a Protein Misfolding Cyclic Amplification assay (PMCA) (*Saborio et al., 2001*) where hamster brain homogenate is used as a source of PrP^C and conversion factors as follows.

a) Brain preparation:

Brains from healthy Syrian golden hamsters healthy or infected with the adapted scrapie strain 263 K are obtained after decapitation and immediately frozen in dry ice and kept at –80° until used. Brains are homogenized in PBS containing protease inhibitors (Complete™ cocktail from Boehringer Mannheim) at a 1x final concentration. Detergents (0.5% Triton X-100, 0.05% SDS, final concentrations) are

added and samples clarified with low speed centrifugation (10000 g) for 1 min, using an Eppendorf centrifuge (model 5415).

Dilutions (3200-fold and 12800-fold) of the scrapie brain homogenate are added directly to the healthy brain homogenate to trigger prion replication. 60µl of these mixtures are frozen immediately and another 60µl are incubated at 37°C with agitation. Each hour a cycle of sonication (5 pulses of 1sec each) is done using a microsonicator (Bandelin Electronic, model Sonopuls) with the probe immersed in the sample and the power setting fixed at 40%. These cycles are repeated 10 times.

b) PMCA signal in presence and absence of a cholesterol-depleting agent:

Under these conditions a dramatic increase in the amount of PrP^{Sc} signal is observed after 10 cycles of PMCA (Figure 1, lanes 2 and 3). When normal brain homogenate is treated during 30 min with 10 and 20mM (but not 5 mM) methyl-β–cyclodextrin (MβCD) a complete inhibition of prion replication is observed (Figure 1, lanes 6-9) as obtained in mouse models in cell cultures and in vitro, indicating that cholesterol depletion has a detrimental effect on prion replication *(Taraboulos et al., 1995)*.

c) PMCA signal in presence of apolipoproteins

Purified delipidated human ApoB (Figure 2A) and human Apo E (Figure 2B) are respectively added to the PMCA preparation without cyclodextrin at different concentrations (8 and 16 µg for hApo B) and (1 and 10 µg for hApo E). Samples are incubated for 30 min at 4°C and thereafter half of each sample is frozen and the other half subjected to PMCA cycles.

An increase in prion replication in vitro is observed at both 3200-fold and 12800-fold dilutions of scrapie brain homogenate for both Apolipoprotein B and Apolipoprotein E.

In contrast, addition of Apolipoprotein J (at concentrations of 1, 2 and 4 µg), an Apolipoprotein component of HDL, has no effect on PMCA signal (Figure 2C).

These data show the effect of Apolipoprotein B and E implicated in the prion conversion.

EXAMPLE 2: *In vitro* prion replication in lipid rafts from prion infection sensitive cells by PMCA:

The mouse neuroblastoma cell line N2a is used for their capability to be infected with PrPSc. Baron et al., 2002 and Enari et al., 2001 have shown that prion infection sensitive and prion infection resistant N2a sub-lines exist. Lipid rafts from the prion infection sensitive line are isolated and used as a substrate for PMCA assay. The effect of an apolipoprotein B antagonist on prion conversion is studied through the ability of apolipoprotein B antagonist to inhibit the prion replication ability of prion infection sensitive N2a cell lines.

a) N2a cell preparation:

Sub-clones of the parental mouse neuroblastoma N2a cell line are derived from single cells by limit dilution. A growing culture (Dulbecco's Modified Eagles Medium (DMEM Gibco # 41966-029), containing 10% fetal calf serum (FCS) and supplemented with 2mM, L-glutamate and standard antibiotics (penicillin and streptomycin)) is diluted to a density of 5 cells/ml and 100µl is transferred to individual wells of a 96 well plate and cultured for 1 week.

The individual cultures are examined microscopically to determine those wells which contained a single focus of growing cells. The single cell derived cultures are then transferred to 24 well plates and serially passaged every 3-4 days at 1:15 dilution to maintain stocks. A total of 63 cultures are isolated and all tested for sensitivity to infection by the RML strain of PrP^{Sc}. To do this, 4µl of a 10% late stage infected brain extract is added per well of newly passaged cells, and the cultures are left for a further 4 days to reach 100 % confluence. Cells were serially passaged thereafter in the absence of PrP^{Sc}. Tests showed that all trace of the initial innoculum disappeared by passage 4.

At this and later passages individual cultures are tested for the presence of PrP^{Sc}.

b) Prion infection resistant cell isolation by Cell culture dot blotting:

The presence of PrP^{Sc} in the 63 individual cell cultures is tested by cell culture dot blotting procedure in which lysis and proteinase K (PK) digestion are carried out directly in the culture dish. PK resistant PrP^{Sc} is detected by dot blotting to PVDF membranes and exposure to anti-PrP antibody as follows:

Infected cells are grown for 3-4 days in 24-well plates and washed once with PBS. 40µl DNaseI (1000U/ml in H20) is added to each well at room temperature for 5min, followed by 40µl proteinase K solution (20µg/ml in 100mMTris/HCl pH 7.4, 300mM NaCl, 1% Triton-X100, 1% sodium deoxycholate). Plates are incubated at 37°C for 1hr with gentle agitation. Proteinase K digestion is stopped by addition of 2µl of a solution containing 80µg/ml PMSF, 10mmTris-HCl pH 8.0 and 1mg/ml bromophenol blue. 20µl aliquots are spotted onto PVDF membranes equilibrated with a degasses solution containing 192mM Glycine, 25mM Tris, 20% methanol. Membranes are then transferred to 3M guanidine Thiocyanate, 10mM Tris HCL pH 8.0 for 10min to denature proteins, rinsed in water and processed as for Western blotting using anti-PrP 6H4 (Prionics). Non-specific binding is blocked by incubation with 5% milk dissolved in PBS for 1hr. The membrane is then exposed to specific primary antibody anti-PrP 6H4, followed by HRP-conjugated secondary antibody each diluted as appropriate in PBS, 0.3% Tween 20. Western blots are developed by ECL™ (Amersham) as directed according to the provider instructions.

The chemiluminescence signal from membranes is then analyzed directly using the Kodak Imagestation 440CF. The luminescence signal in each condition was normalized for possible differences in cell growth. Total protein content of a parallel lysate untreated with proteinase K is determined using the BCA assay (Biorad) and results are expressed as intensity/µg protein.

Of the 63 sub-clones analysed, 9 were found to be capable of replicating PrP^{Sc}, albeit with differing efficiencies (Figure 3). The remaining 54 sub-clones were resistant to infection. The most highly prion infection sensitive cell lines were selected for further study together with several prion infection resistant sub-clones with similar morphologies and doubling times. We have have selected two of these cell lines: #23, a prion infection resistant clone, and #60 a prion infection sensitive clone.

These two cell lines have been maintained in culture for over 1 year and have been infected with RML in many different occasions throughout this period: on every occasion sub-clone #60 was highly infectable whereas sub-clone #23 was totally resistant. Prion infection sensitive sub-clones could be maintained as a chronically infected cell culture by serial passaging at 1:15 or 1:20 dilution every 3 or 4 days respectively.

No evident morphological differences by microscopy were observed between the resistant or sensitive cells or between non-infected and infected cells.

To validate the clinical relevance of this cellular model of PrP relication, extracts of chronically infected N2a cells, or buffer alone, were injected into the hippocampus of normal mice by stereotactic injection. Injection of N2a extracts resulted in onset of clinical symptoms of scrapie after 140 days and premature death whereas mock injection had no effect on mouse physiology or life span. This indicates that the cell based model for prion replication using prion infection sensitive N2a cells generates infectious PrP scrapie, confirming that the conversion of PrP in cells is a good model for the process which occurs in vivo.

c) Lipid rafts isolation:

Procedures for isolating lipid rafts based on resistance to solubilization in cold Triton X-100 followed by flotation on sucrose gradients have been described by numerous laboratories *(Simons et al., 2000; Hooper et al., 1999)*. Lipid rafts from the two cell lines selected above are carried out as follows:

Subconfluent cultures of N2a cells are washed in PBS and collected by centrifugation 1000 x g for 5min. Typically 3 x 15cm dishes are pooled equivalent to approximately 8 $x10^7$ cells. The cell pellet is re-suspended in 1ml ice cold raft buffer (1% Triton in PBS, containing 10μM copper sulphate and a cocktail of complete protease inhibitors (Boehringer)). Cells are disrupted by seven passages through a 22G needle followed by incubation for 30 min at 4°C with gentle agitation. 2 volumes 60% sucrose in PBS is added and the lysate is transferred to a SW41 centrifuge tube. The lysate is carefully overlaid with 7 ml 35% sucrose and 1ml 15% sucrose both in PBS and centrifuged 20hr at 35,000 RPM. The lipid rafts are recovered in the top 1ml of the gradient. Membranes are concentrated by addition of 10 volumes cold PBS and centrifugation at 100,000g for 2hr. Alternatively for 2D gel electrophoresis, proteins from the lipid raft fraction are recovered by precipitation in the presence of 5 vol acetone for 2hr at –80°C. Acetone precipitates are collected by centrifugation 14000g 20min and washed twice in 70% ethanol.

In both sensitive and resistant cells around 1-2% of protein in the total lysate is recovered in the bouyant raft fraction. As shown by Western blotting (Figure 4A) while PrP is barely detectable in the total cell extract, it is highly enriched in rafts leaving the sample layer totally depleted of PrP following centrifugation.

Prion infection sensitive clone #60 and the prion infection resistant clone #23 are compared by westerm blotting with anti-PrP (Figure 4B). Three different independent pairs of raft preparations each containing 5µg total raft proteins are re-probed with anti-actin antibody which confirms the uniformity of PrP protein loading (Figure 4C).

The results indicate that the level of PrP in the lipid raft preparations from the two cell types is indistinguishable. Moreover the distribution between non-glycosylated mono- and di-glycosylated isoforms as well as the segregation to the detergent resistant membrane fraction shown in Figure 4A is identical suggesting that none of these factors are likely to be responsible for the differing phenotypes.

PrP cDNA was amplified by RT-PCR from both cell lines as follows:
Total RNA of N2a cells is prepared using Trizol (Gibco) and the mouse PrP cDNA is reversed transcribed with Omniscript (Qiagen) using the protocol supplied by the manufacturer. The specific primer for cDNA synthesis is 5' TCAATTGAAAGAGCTACAGGTG 3'. The prion cDNA is amplified using standard PCR conditions in the presence of primers 5' ACCAGTCCAATTTAGGAGAGCC 3' (top strand) and 5' AGACCACGAGAATGCGAAGG 3' (bottom strand). The PCR product was completely sequenced in the automated ABI3700 using the reagents and the protocol supplied by the manufacturer.

These data revealed that PrP mRNA is wild type in both cases and that both carry a Methionine at position 129, which in humans is the site for a frequent polymorphism.

Therefore, the expression levels, glycosylation patterns, intracellular localisation and primary sequences of PrP^{C} in both cell types is indistinguishable and thus that other cellular factors are responsible for the differential response to PrP^{Sc}.

d) *In vitro* cyclic amplification of protein misfolding (PMCA) in lipid rafts from prion infection sensitive cells:

Lipid rafts obtained at §c are isolated from prion infection sensitive sub-clones, #60 sub-clones, collected by centrifugation as described above and re-suspended in PMCA conversion buffer at a concentration of 2-2.5 mg/ml (PBS containing final concentration of 300mM NaCl, 0.5% Triton X100, 0.05% SDS).

A 10% extract of RML-infected mouse brain homogenate is added directly to the rafts preparation at a dilution of 1:100 based on protein content and aliquots of the mixture are either frozen immediately, incubated for 15hr at 37°C or subjected to 15 cycles of PMCA (5 x 0.1 second pulses of sonication followed by incubation at 37 °C for 1hr).

Aliquots of 20µl sample are then treated with 10 µg /ml Proteinase K for 1hr at 37°C. Lipids are removed by precipitating PK-resistant proteins with 5 vol acetone for 2hr at –80°C. Acetone precipitates are collected by centrifugation 14000g 20 min, washed twice in 70% ethanol analysed by Western blotting with 6H4 anti-PrP (Figure 5).

Compared to the mixture without PK treatment (lanes 1 and 5) all digested samples show a shift in molecular weight characteristic of the N-terminally truncated PK resistant form PrP_{27-30}. It should be noted that the 6H4 antibody also has low level cross reactivity with PK which migrates at 30kDa, close to the di-glycosylated form of PK-digested PrP. Analysis of the data with this in mind shows that the initial level of PK-resistant PrP derived from the diluted brain extract, which is present in the non-amplified mixtures, is barely detectable under these conditions (lanes 2).

A slight increase in signal is seen when the prion infection sensitive (#60) DRM is incubated at 37 °C for 15 hr (lane 3 from #60), however the most dramatic increase in PK-resistant PrP is seen when this sample is subjected to 15 cycles of PMCA (lane 4 from #60). This indicates that all factors required for conversion of PrP^{C} to PrP^{Sc} are resident in the lipid rafts from the prion infection sensitive N2a cells. Interestingly, in the parallel analysis in which the DRM from the prion infection resistant cell line #23 was used, no amplification in vitro was observed (lane 4 from #23) indicating that the capacity of the lipid rafts to convert the prion protein in vitro reflects the activity observed in the intact cells.

e) Effect of antibody raised against apolipoprotein B on prion replication by prion infection sensitive N2a cells:

Chronically infected sensitive cells were cultured in 24-well dishes in the presence of a goat polyclonal antibody raised against human Apo B (Chemicon) at increasing concentrations from 0 to 2 mg/ml in DMEM Gibco # 41966-029, containing 1 x B27 supplements (Gibco #17504-044) and standard antibiotics (penicillin and streptomycin)

A parallel series of cultures was incubated in the presence of the same concentration range of total IgG from a naïve goat. The results show that concentrations of anti-hApoB antibody above 0.5mg/ml result in progressive inhibition of PrP replication as revealed by quantitative dot blotting (Figure 6).

These data show the role of Apolipoprotein B in the prion conversion.

EXAMPLE 3: Proteomics analysis of lipid rafts of prion infection resistant and sensitive cells:

Since the two cell preparations are indistinguishable in terms of their PrP content a more complete protein comparison using 2D gel electrophoresis was performed to show differences in other proteins that might underline the difference in conversion activity between the two sub-clones.

2D gel preparations are prepared as follows:

Acetone precipitated proteins (see §c) are re-suspended in 20µl 1% SDS, 0.23% DTT and heated to 95°C for 5min. After the preparation is cooled to room temperature, 25µl of a solution (9M urea, 4% CHAPS, 65mM DTT, 35mM Tris base) is added.

Fifteen minutes later, 85µl of a solution containing 7M urea, 2M thiourea, 4% CHAPS, 100mM DTT is further added to the mixture. After a further 15 min, non-solubilized material is removed by centrifugation at 14000 RPM during 5min and the supernatant is applied directly to a 7cm IPG strip and left to re-hydrate overnight. For IEF the voltage is progressively increased from 300V to 3.5kV and electrophoresed for a total of 20kVh. Proteins are resolved in the second dimension using single well 4-12% gradient gels (Novex) and stained using the silver express kit (Invitogen) according to the instructions supplied.

Analysis by 2D gels reveals the fraction of protein that is recovered in the lipid rafts (approximately 1-2% protein in the N2a cell lysate) as a reproducible subset of total cell proteins in which several hundred species can be visualized following silver staining (Figure 7A and B).

The 2D patterns are compared between preparations isolated from the prion infection sensitive and resistant cells. The analysis is focused on several proteins identified in the basic range of the gel, which are more abundant in DRMs from prion infection resistant cells (arrows in Figure 7C and D).

Following preparative scale electrophoresis, the two proteins indicated by arrows are excised and processed for MS sequencing. From both proteins an identical tryptic peptide is found with a monoisotopic mass of 1234.6. The N-terminal sequence of this tryptic peptide is: ENFAGEATLQR (SEQ ID NO: 3). All amino acids in the peptide are identified in the MS/MS spectrum of doubly charged precursor ion at m/z 618.30 And through its Mascot analysis.

Database searching identified this protein unambiguously as Apolipoprotein B (Apo B). Since the molecular weight of full length Apo B is in excess of 500 kDa while these two spots migrate with estimated molecular weights of 40kDa and 30kDa, we presume that the latter are fragments generated either in the cell or during sample preparation. The sequence corresponds to amino acids 3548-3558 of the human Apo B protein, which is present only in ApoB-100 and not in the truncated ApoB-48 form.

These data suggest that fragments of a molecular weight of or about 30 to 40 kDa comprising the sequence of SEQ ID NO: 3 may have a role in the prion conversion pathway.

EXAMPLE 4: Binding and internalisation of fluorescent LDL receptor by resistant and sensitive cells:

N2a subclones #23 (prion infection sensitive) and #60 (prion infection resistant) were cultured in 24 well plates in standard DMEM medium containing 10% FCS for 2 days then transferred to the same medium (300 µl) containing 1% FCS for 1hr. To visualize cell surface binding, plates were placed on ice to inhibit endocytosis and 3µl fluorescent DiI-LDL (Molecular Probes) was added for 30 min.

LDL-binding was visualized by fluorescence microscopy. To study LDL uptake by each of the sub-clones, cells were incubated at 37 C with 3µl DiI-LDL for 2h prior to microscopic examination.

Control cultures were incubated in parallel with DiI-coupled *acetylated* LDL which does not bind the LDL receptor or with Hoechst to visualize cell nuclei.

The binding or uptake of fluorescent DiI-LDL is similar for prion infection resistant and prion infection sensitive cells, suggesting that the level of the LDL receptor between these two cell types is similar.

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US 5,948,763;
US 6,022,683;
US 6,197,972;
US 6,355,610;
US 6,552,922;
US 20020128175;
US 20020155426;
WO 97/14437;
WO 99/15159;
WO 0168710;
WO 0204954;
WO 02065133;
WO 03002533;
WO 03005037;
WO 03045921;
WO 2004043403.

Claims

1. Use of a peptide or a protein selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof, in an assay for the detection of the formation of PrP^{sc} in a sample.

2. Use of a peptide or a protein selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof, in a screening assay for identifying compounds that modulate the conversion of PrP^{c} into PrP^{sc}.

3. Use according to claims 1 or 2 wherein the peptide or protein is selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof, which forms a complex with the LDL receptor.

4. Use according to any of the preceding claims wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay.

5. Use according to any of the preceding claims wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using normal brain homogenate as a source of normal PrP^{C} and substrate.

6. Use according to claims 1 to 4 wherein the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using lipid rafts from infection sensitive neuroblasma cell line N2a as a source of normal PrP^{C} and substrate.

7. Use according to any of the preceding claims wherein the protein is Apolipoprotein B.

8. Use according to any of the preceding claims wherein the protein is Apolipoprotein B, the assay is a Protein Misfolding Cyclic Amplification (PMCA) assay using lipid rafts from infection sensitive neuroblasma cell line N2a as a source of normal PrP^{C} and substrate.

9. Use of a modulator of a protein or a peptide, wherein the protein is selected from from Apolipoprotein B and a fragment thereof, for the preparation of a pharmaceutical composition for the treatment of a prion disease.

10. Use according to claim 9 wherein the modulator is an antibody raised against Apolipoprotein B or a fragment thereof.

11. Use according to any of the preceding claims wherein the peptide or the protein contains the sequence of SEQ ID NO: 3.

12. Use according to any of the preceding claims wherein the peptide or the protein is of a molecular weight selected from 30 and 40 kDa and which sequence is selected from fragments of Apolipoprotein B taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

13. Use according to any one of claims 9 to 12 wherein the prion disease is bovine spongiform encephalopathy (BSE).

14. Use according to any one of claims 9 to 12 wherein the prion disease is a Creutzfeld-Jacob Disease (CJD).

15. A method for the diagnosis or detection of a prion disease within a subject suspected of suffering from such a disease which comprises (i) contacting a sample from said subject with a peptide or a protein selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof; (ii) contacting the mixture obtained in step (i) with PrP^{C} or PrP^{C} containing mixtures; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

16. A method of determining a marker that predisposes a subject to a prion disease, comprising (i) measuring a level of a protein selected from Apolipoprotein B; a

fragment thereof; in said sample; and (ii) correlating said level of protein obtained in said measuring step with the occurrence of a prion disease.

17. A method according to any one of claims 15 to 16 wherein the prion disease is bovine spongiform encephalopathy (BSE).

18. A method according to any one of claims 15 to 16 wherein the prion disease is a Creutzfeld-Jacob disease.

19. A method for the detection of PrP^{Sc} within a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof; (ii) contacting sample obtained in (i) with PrP^{C} or PrP^{C} containing mixtures; and (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

20. A method for identifying, in a sample, a compound which modulates the transition of PrP^{C} into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof; (a) in the presence of said modulatory compound and (b) in the absence of said compound; (ii) contacting the mixtures obtained in step (i) a and (i) b with PrP^{C} or PrP^{C} containing mixtures; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said modulatory compound and (b) in the absence of said modulatory compound.

21. A method according to any one of claims 15 to 20 wherein the peptide or the protein contains the sequence of SEQ ID NO: 3.

22. A method according to any one of claims 15 to 21 wherein the peptide or the protein is of a molecular weight selected from 30 and 40 kDa and which sequence is selected from fragments of Apolipoprotein B taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

23. An assay for the detection of PrP^{Sc} in a sample, which assay comprises (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof; (ii) contacting the mixture obtained in step (i) with PrP^{C} or PrP^{C} containing mixtures; (iii) determining the presence and/or amount of PrP^{Sc} in said sample.

24. A screening assay for identifying a compound which modulates the transition of PrP^{C} into PrP^{Sc} comprising: (i) contacting said sample with a peptide or a protein selected from Apolipoprotein B; a fragment thereof; Apolipoprotein E and a fragment thereof; (a) in the presence of said modulatory compound and (b) in the absence of said modulatory compound; (ii) contacting the mixtures obtained in step (i) a and (i) b with PrP^{C} or PrP^{C} containing mixtures; and (iii) determining the amount of PrP^{Sc} (a) in the presence of said compound and (b) in the absence of said modulatory compound.

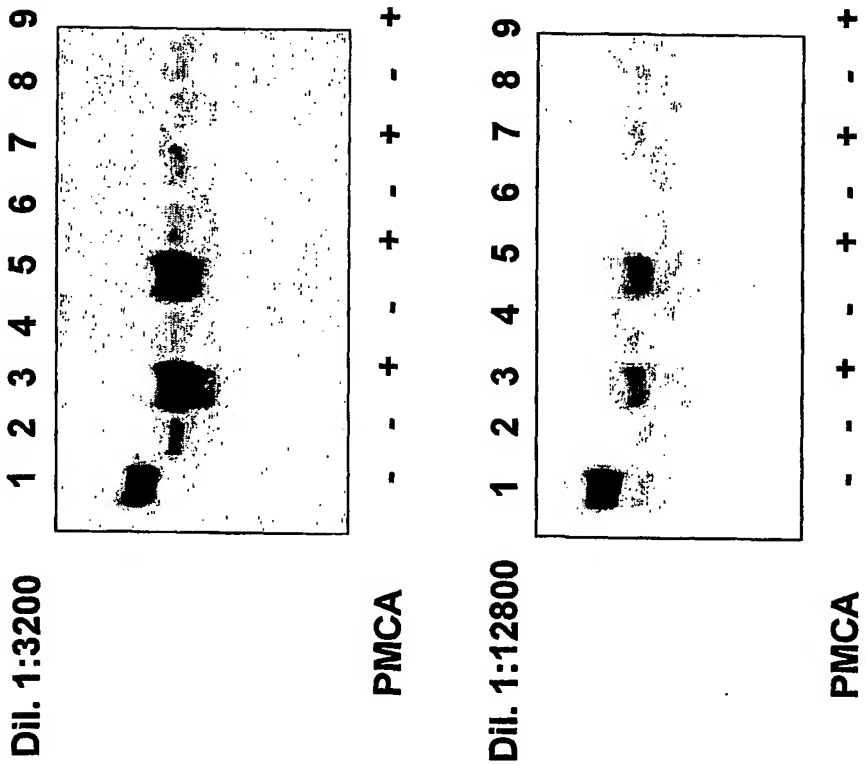
25. An assay according to any one of claims 23 to 24 wherein the peptide or the protein contains the sequence of SEQ ID NO: 3.

26. An assay according to any one of claims 23 to 25 wherein the peptide or the protein is of a molecular weight selected from 30 and 40 kDa and which sequence is selected from fragments of Apolipoprotein B taken between positions 3201-3558, 3548-3905, 3201-3905, 3291-3558, 3548-3815 and 3291-3815.

27. A diagnostic kit for use in an assay according to claims 23 to 26, comprising a probe for receiving a sample and a peptide or a protein selected from Apolipoprotein B and a fragment thereof.

28. An apparatus for use in a method according to any one of claims 15 to 22 or an assay according to any one claims 23 to 26.

Figure 1



Lanes	MβCD concentration, mM
2, 3	0
4, 5	5
6, 7	10
8, 9	20

Figure 2A

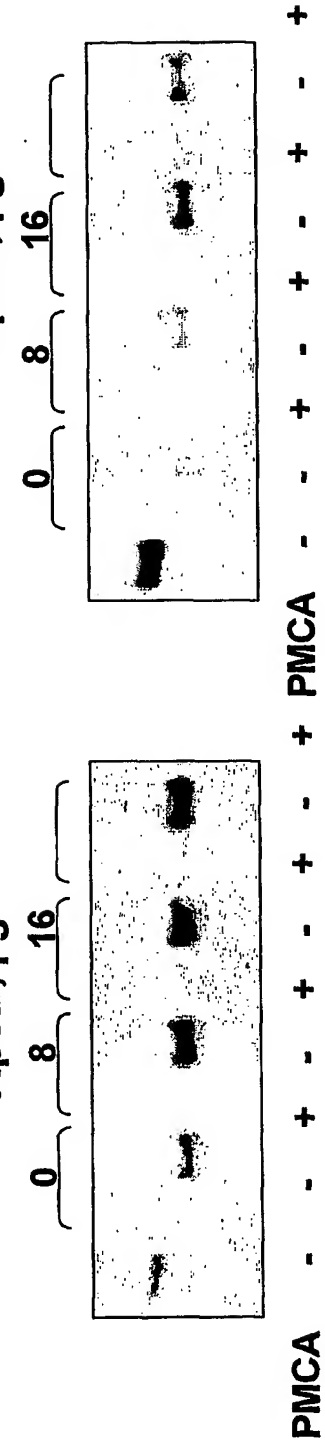


Figure 2B

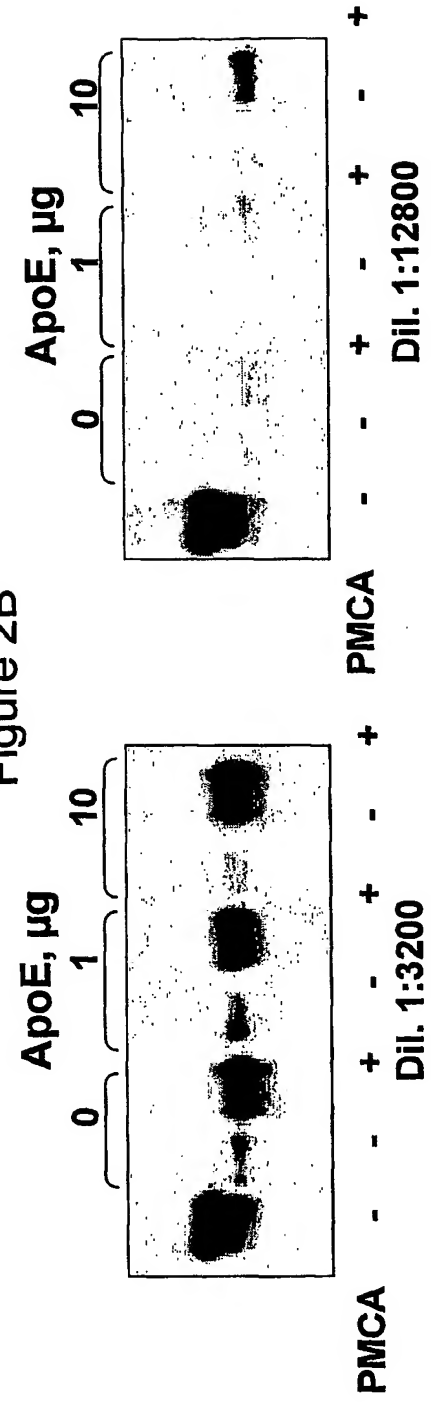
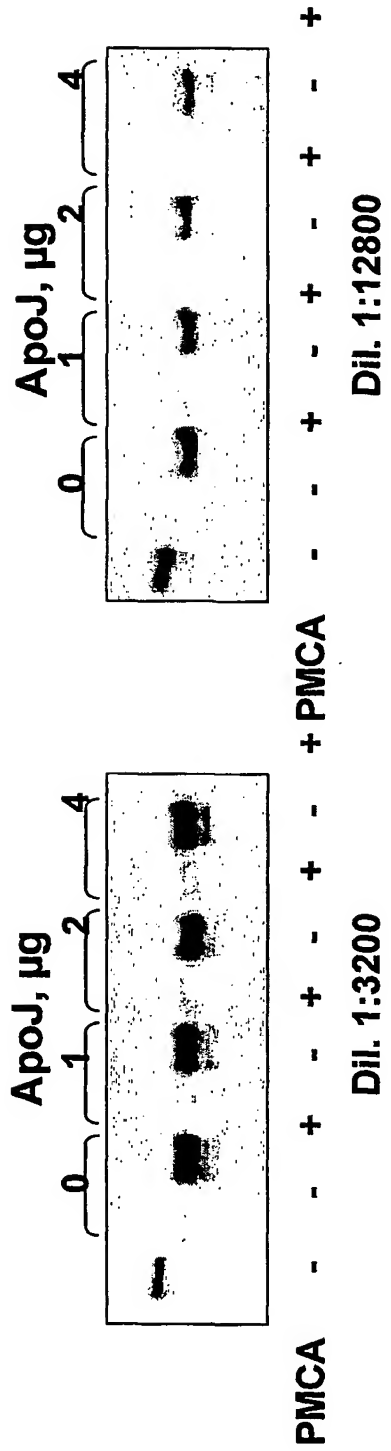


Figure 2C



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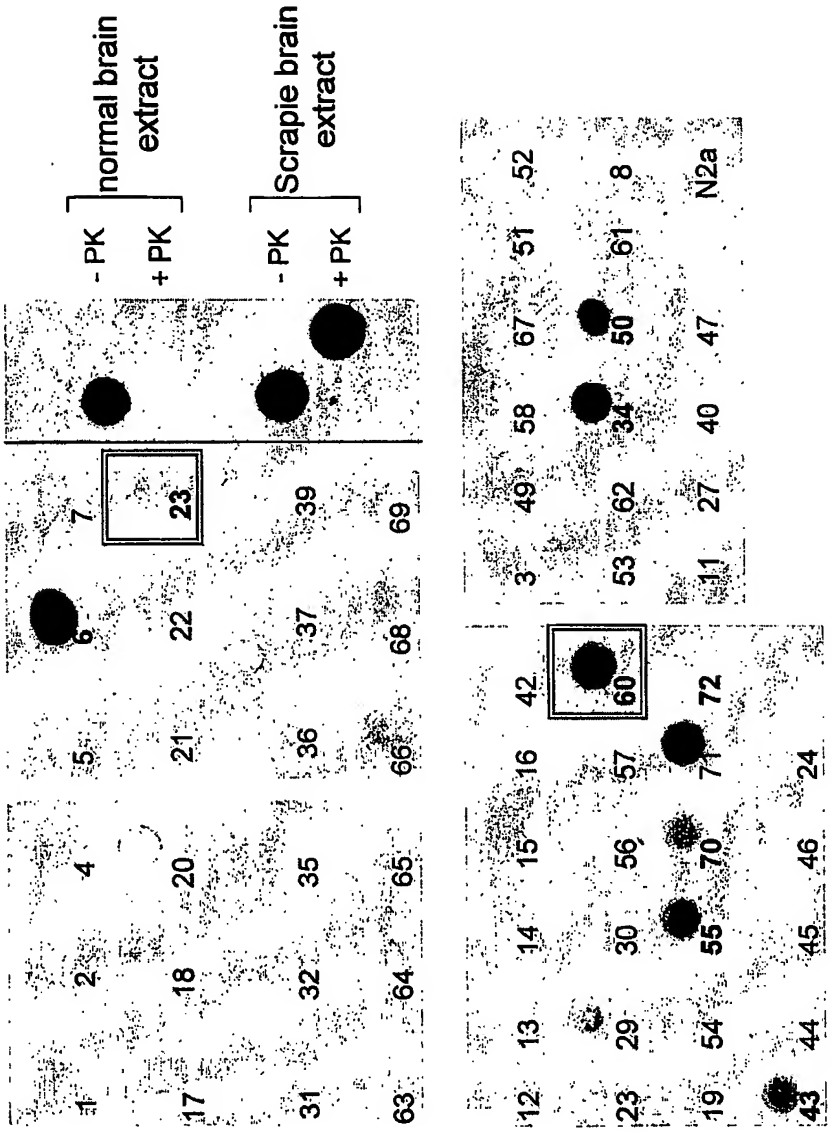

controls
normal brain extract
Scrapie brain extract
- PK
+ PK
- PK
+ PK
N2a

Figure 4

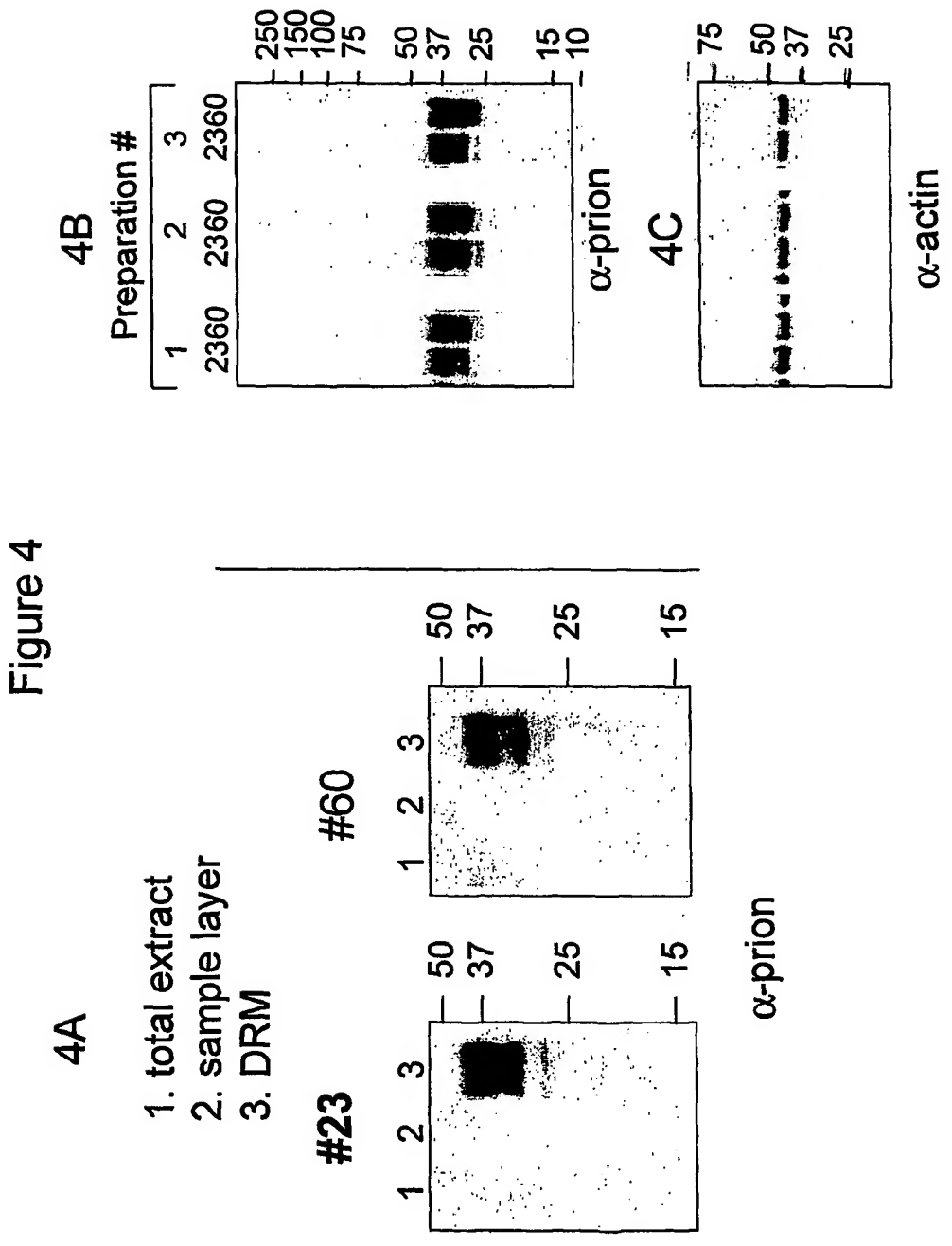
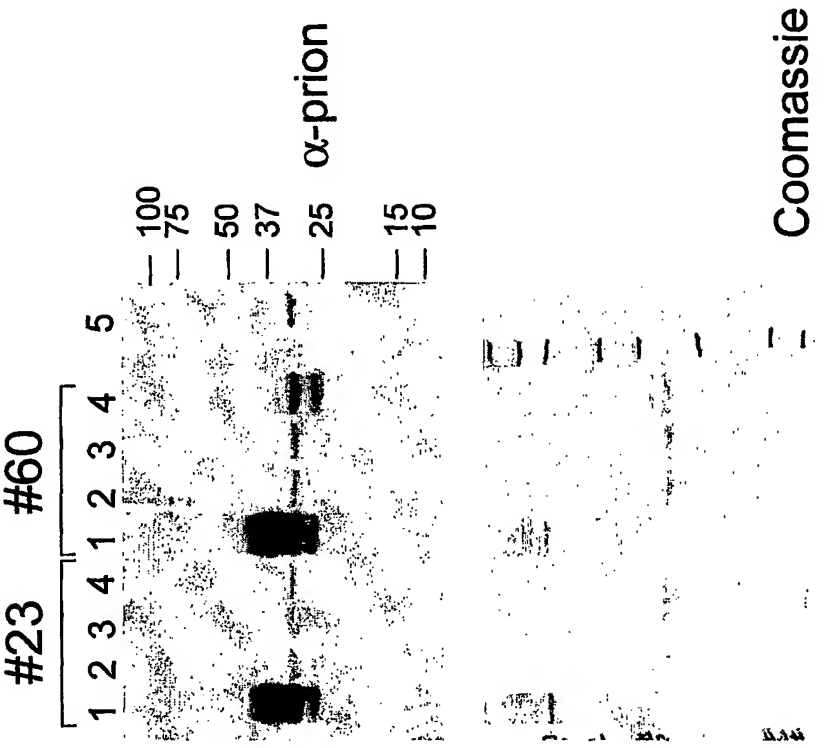
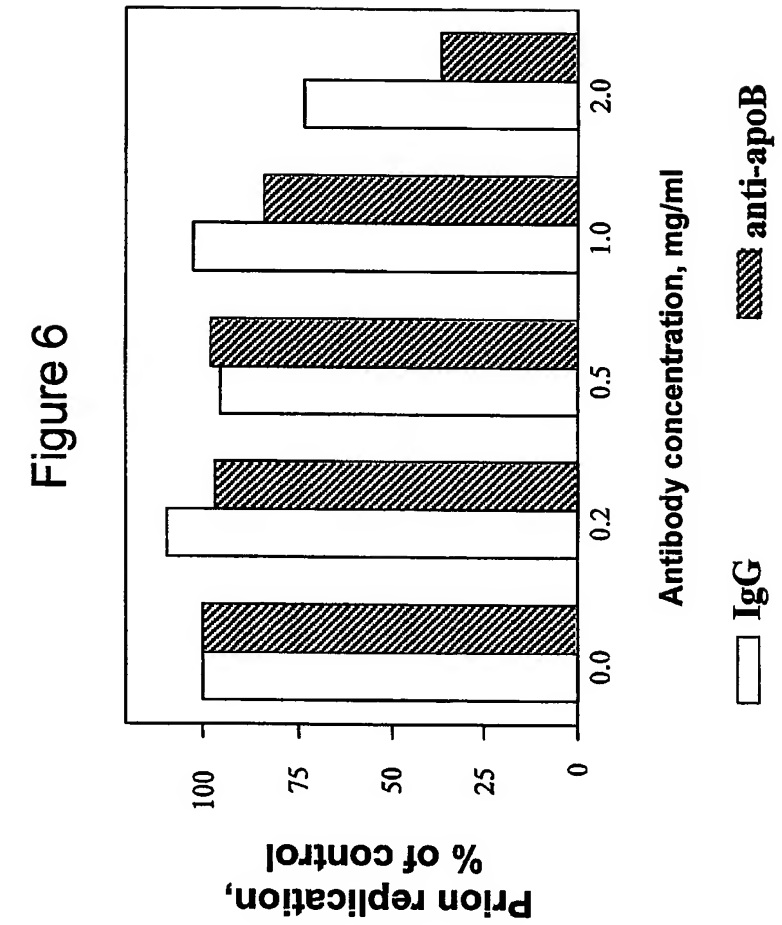

4A
1. total extract
2. sample layer
3. DRM
#23
#60
1 2 3
50
37
25
15
α-prion
4B
Preparation #
1 2360
2 2360
3 2360
250
150
100
75
50
37
25
15
10
α-prion
4C
75
50
37
25
α-actin

Figure 5



1. frozen - w/o PK
2. frozen - PK digested
3. 37°C 15h - PK digested
4. PMCA 15 cycles - PK digested

Figure 6


Prion replication,
% of control
100
75
50
25
0
0.0
0.2
0.5
1.0
2.0
Antibody concentration, mg/ml
IgG
anti-apoB

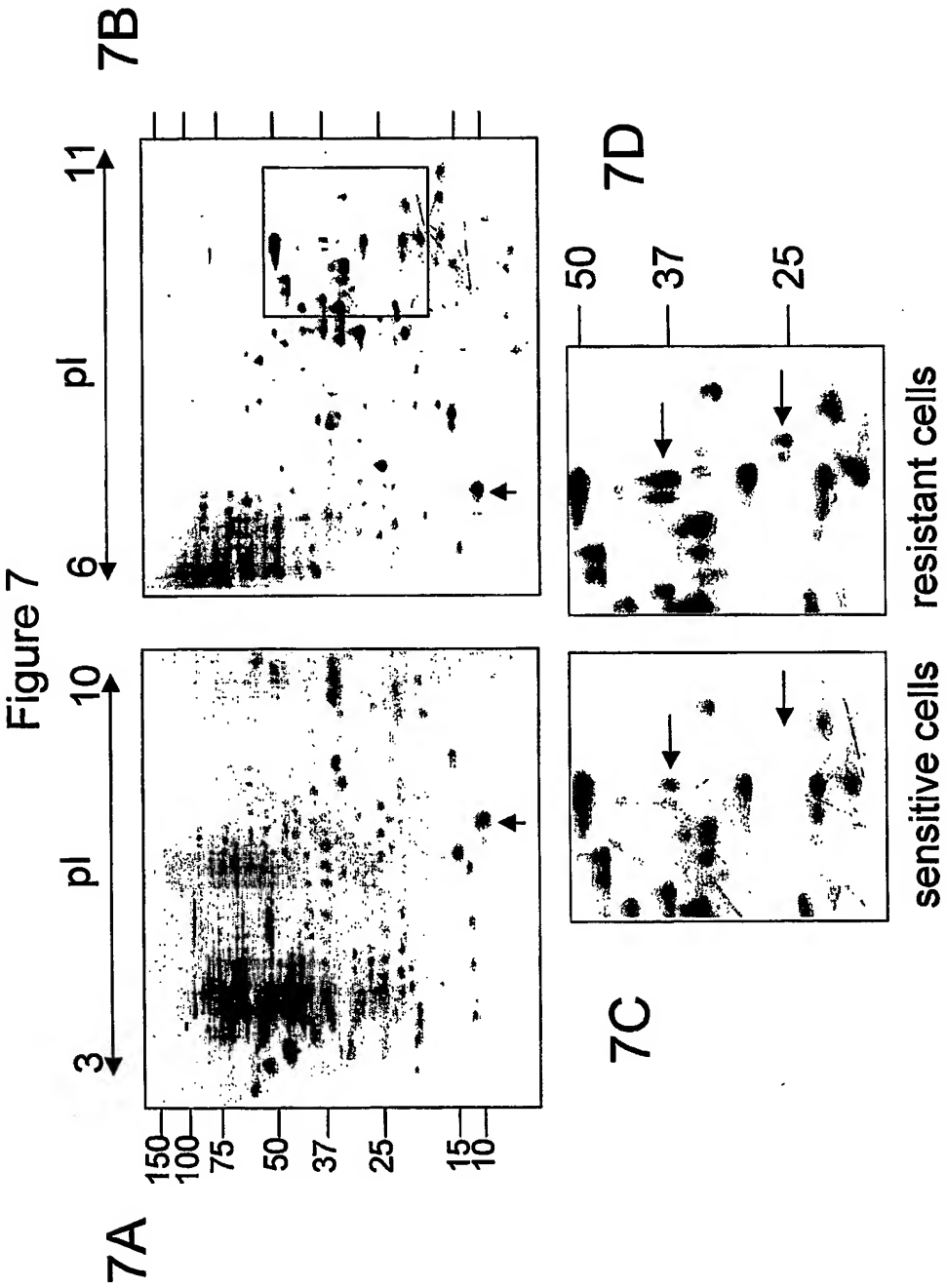

7A
7B
7C
7D
pI
3
10
6
11
150
100
75
50
37
25
15
10
50
37
25
sensitive cells
resistant cells

Figure 7

SEQUENCE LISTING

<110> APPLIED RESEARCH SYSTEMS ARS HOLDING N.V.

<120> USE OF PRION CONVERSION MODULATING AGENTS

<130> WO/845

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            20                  25                  30

Glu Asn Val Ser Leu Val Cys Pro Lys Asp Ala Thr Arg Phe Lys His
        35                  40                  45

Leu Arg Lys Tyr Thr Tyr Asn Tyr Glu Ala Glu Ser Ser Ser Gly Val
    50                  55                  60

Pro Gly Thr Ala Asp Ser Arg Ser Ala Thr Arg Ile Asn Cys Lys Val
65                  70                  75                  80

Glu Leu Glu Val Pro Gln Leu Cys Ser Phe Ile Leu Lys Thr Ser Gln
                85                  90                  95

Cys Thr Leu Lys Glu Val Tyr Gly Phe Asn Pro Glu Gly Lys Ala Leu
            100                 105                 110

Leu Lys Lys Thr Lys Asn Ser Glu Glu Phe Ala Ala Ala Met Ser Arg
        115                 120                 125

Tyr Glu Leu Lys Leu Ala Ile Pro Glu Gly Lys Gln Val Phe Leu Tyr
    130                 135                 140

Pro Glu Lys Asp Glu Pro Thr Tyr Ile Leu Asn Ile Lys Arg Gly Ile
145                 150                 155                 160

Ile Ser Ala Leu Leu Val Pro Pro Glu Thr Glu Glu Ala Lys Gln Val
```

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                165                 170                 175

Leu Phe Leu Asp Thr Val Tyr Gly Asn Cys Ser Thr His Phe Thr Val
            180                 185                 190

Lys Thr Arg Lys Gly Asn Val Ala Thr Glu Ile Ser Thr Glu Arg Asp
        195                 200                 205

Leu Gly Gln Cys Asp Arg Phe Lys Pro Ile Arg Thr Gly Ile Ser Pro
    210                 215                 220

Leu Ala Leu Ile Lys Gly Met Thr Arg Pro Leu Ser Thr Leu Ile Ser
225                 230                 235                 240

Ser Ser Gln Ser Cys Gln Tyr Thr Leu Asp Ala Lys Arg Lys His Val
                245                 250                 255

Ala Glu Ala Ile Cys Lys Glu Gln His Leu Phe Leu Pro Phe Ser Tyr
            260                 265                 270

Asn Asn Lys Tyr Gly Met Val Ala Gln Val Thr Gln Thr Leu Lys Leu
        275                 280                 285

Glu Asp Thr Pro Lys Ile Asn Ser Arg Phe Phe Gly Glu Gly Thr Lys
    290                 295                 300

Lys Met Gly Leu Ala Phe Glu Ser Thr Lys Ser Thr Ser Pro Pro Lys
305                 310                 315                 320

Gln Ala Glu Ala Val Leu Lys Thr Leu Gln Glu Leu Lys Lys Leu Thr
                325                 330                 335

Ile Ser Glu Gln Asn Ile Gln Arg Ala Asn Leu Phe Asn Lys Leu Val
            340                 345                 350

Thr Glu Leu Arg Gly Leu Ser Asp Glu Ala Val Thr Ser Leu Leu Pro
        355                 360                 365

Gln Leu Ile Glu Val Ser Ser Pro Ile Thr Leu Gln Ala Leu Val Gln
    370                 375                 380

Cys Gly Gln Pro Gln Cys Ser Thr His Ile Leu Gln Trp Leu Lys Arg
385                 390                 395                 400

Val His Ala Asn Pro Leu Leu Ile Asp Val Val Thr Tyr Leu Val Ala
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```
Leu Ile Pro Glu Pro Ser Ala Gln Gln Leu Arg Glu Ile Phe Asn Met
            420                 425                 430

Ala Arg Asp Gln Arg Ser Arg Ala Thr Leu Tyr Ala Leu Ser His Ala
        435                 440                 445

Val Asn Asn Tyr His Lys Thr Asn Pro Thr Gly Thr Gln Glu Leu Leu
    450                 455                 460

Asp Ile Ala Asn Tyr Leu Met Glu Gln Ile Gln Asp Asp Cys Thr Gly
465                 470                 475                 480

Asp Glu Asp Tyr Thr Tyr Leu Ile Leu Arg Val Ile Gly Asn Met Gly
                485                 490                 495

Gln Thr Met Glu Gln Leu Thr Pro Glu Leu Lys Ser Ser Ile Leu Lys
            500                 505                 510

Cys Val Gln Ser Thr Lys Pro Ser Leu Met Ile Gln Lys Ala Ala Ile
        515                 520                 525

Gln Ala Leu Arg Lys Met Glu Pro Lys Asp Lys Asp Gln Glu Val Leu
    530                 535                 540

Leu Gln Thr Phe Leu Asp Asp Ala Ser Pro Gly Asp Lys Arg Leu Ala
545                 550                 555                 560

Ala Tyr Leu Met Leu Met Arg Ser Pro Ser Gln Ala Asp Ile Asn Lys
                565                 570                 575

Ile Val Gln Ile Leu Pro Trp Glu Gln Asn Glu Gln Val Lys Asn Phe
            580                 585                 590

Val Ala Ser His Ile Ala Asn Ile Leu Asn Ser Glu Glu Leu Asp Ile
        595                 600                 605

Gln Asp Leu Lys Lys Leu Val Lys Glu Ala Leu Lys Glu Ser Gln Leu
    610                 615                 620

Pro Thr Val Met Asp Phe Arg Lys Phe Ser Arg Asn Tyr Gln Leu Tyr
625                 630                 635                 640

Lys Ser Val Ser Leu Pro Ser Leu Asp Pro Ala Ser Ala Lys Ile Glu
                645                 650                 655
```

```
Gly Asn Leu Ile Phe Asp Pro Asn Asn Tyr Leu Pro Lys Glu Ser Met
            660                 665                 670

Leu Lys Thr Thr Leu Thr Ala Phe Gly Phe Ala Ser Ala Asp Leu Ile
        675                 680                 685

Glu Ile Gly Leu Glu Gly Lys Gly Phe Glu Pro Thr Leu Glu Ala Leu
    690                 695                 700

Phe Gly Lys Gln Gly Phe Phe Pro Asp Ser Val Asn Lys Ala Leu Tyr
705                 710                 715                 720

Trp Val Asn Gly Gln Val Pro Asp Gly Val Ser Lys Val Leu Val Asp
                725                 730                 735

His Phe Gly Tyr Thr Lys Asp Asp Lys His Glu Gln Asp Met Val As n
            740                 745                 750

Gly Ile Met Leu Ser Val Glu Lys Leu Ile Lys Asp Leu Lys Ser Lys
        755                 760                 765

Glu Val Pro Glu Ala Arg Ala Tyr Leu Arg Ile Leu Gly Gl u Glu Leu
    770                 775                 780

Gly Phe Ala Ser Leu His Asp Leu Gln Leu Leu Gly Lys Leu Leu Leu
785                 790                 795                 800

Met Gly Ala Arg Thr Leu Gln Gly Ile Pro Gln Me t Ile Gly Glu Val
                805                 810                 815

Ile Arg Lys Gly Ser Lys Asn Asp Phe Phe Leu His Tyr Ile Phe Met
            820                 825                 830

Glu Asn Ala Phe Glu Leu Pro Thr Gly Al a Gly Leu Gln Leu Gln Ile
        835                 840                 845

Ser Ser Ser Gly Val Ile Ala Pro Gly Ala Lys Ala Gly Val Lys Leu
    850                 855                 860

Glu Val Ala Asn Met Gln Ala Gl u Leu Val Ala Lys Pro Ser Val Ser
865                 870                 875                 880

Val Glu Phe Val Thr Asn Met Gly Ile Ile Ile Pro Asp Phe Ala Arg
                885                 890                 895
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Ser Gly Val Gln Met Asn Thr Asn Phe Phe His Glu Ser Gly Leu Glu
            900                 905                 910

Ala His Val Ala Leu Lys Ala Gly Lys Leu Lys Phe Ile Ile Pro Ser
        915                 920                 925

Pro Lys Arg Pro Val Lys Leu Leu Ser Gly Gly Asn Thr Leu His Leu
    930                 935                 940

Val Ser Thr Thr Lys Thr Glu Val Ile Pro Pro Leu Ile Glu Asn Arg
945                 950                 955                 960

Gln Ser Trp Ser Val Cys Lys Gln Val Phe Pro Gly Leu Asn Tyr Cys
                965                 970                 975

Thr Ser Gly Ala Tyr Ser Asn Ala Ser Ser Thr Asp Ser Ala Ser Tyr
            980                 985                 990

Tyr Pro Leu Thr Gly Asp Thr Arg  Leu Glu Leu Glu Leu  Arg Pro Thr
        995                 1000                1005

Gly Glu  Ile Glu Gln Tyr Ser  Val Ser Ala Thr Tyr  Glu Leu Gln
    1010                1015                1020

Arg Glu  Asp Arg Ala Leu Val  Asp Thr Leu Lys Phe  Val Thr Gln
    1025                1030                1035

Ala Glu  Gly Ala Lys Gln Thr  Glu Ala Thr Met Thr  Phe Lys Tyr
    1040                1045                1050

Asn Arg  Gln Ser Met Thr Leu  Ser Ser Glu Val Gln  Ile Pro Asp
    1055                1060                1065

Phe Asp  Val Asp Leu Gly Thr  Ile Leu Arg Val Asn  Asp Glu Ser
    1070                1075                 1080

Thr Glu  Gly Lys Thr Ser Tyr  Arg Leu Thr Leu Asp  Ile Gln Asn
    1085                1090                1095

Lys Lys  Ile Thr Glu Val Ala  Leu Met Gly His Leu  Ser Cys Asp
    1100                1105                 1110

Thr Lys  Glu Glu Arg Lys Ile  Lys Gly Val Ile Ser  Ile Pro Arg
    1115                1120                1125

Leu Gln  Ala Glu Ala Arg Ser  Glu Ile Leu Ala His  Trp Ser Pro
```

```
    1130                1135                1140

Ala Lys  Leu Leu Leu Gln Met  Asp Ser Ser Ala Thr  Ala Tyr Gly
    1145                1150                1155

Ser Thr  Val Ser Lys Arg Val  Ala Trp His Tyr Asp  Glu Glu Lys
    1160                1165                1170

Ile Glu  Phe Glu Trp Asn Thr  Gly Thr Asn Val Asp  Thr Lys Lys
    1175                1180                1185

Met Thr  Ser Asn Phe Pro Val  Asp Leu Ser Asp Tyr  Pro Lys Ser
    1190                1195                1200

Leu His  Met Tyr Ala Asn Arg  Leu Leu Asp His Arg  Val Pro Glu
    1205                1210                1215

Thr Asp  Met Thr Phe Arg His  Val Gly Ser Lys Leu  Ile Val Ala
    1220                1225                1230

Met Ser  Ser Trp Leu Gln Lys  Ala Ser Gly Ser Leu  Pro Tyr Thr
    1235                1240                1245

Gln Thr  Leu Gln Asp His Leu  Asn Ser Leu Lys Glu  Phe Asn Leu
    1250                1255                1260

Gln Asn  Met Gly Leu Pro Asp  Phe His Ile Pro Glu  Asn Leu Phe
    1265                1270                1275

Leu Lys  Ser Asp Gly Arg Val  Lys Tyr Thr Leu Asn  Lys Asn Ser
    1280                1285                1290

Leu Lys  Ile Glu Ile Pro Leu  Pro Phe Gly Gly Lys  Ser Ser Arg
    1295                1300                1305

Asp Leu  Lys Met Leu Glu Thr  Val Arg Thr Pro Ala  Leu His Phe
    1310                1315                1320

Lys Ser  Val Gly Phe His Leu  Pro Ser Arg Glu Phe  Gln Val Pro
    1325                1330                1335

Thr Phe  Thr Ile Pro Lys Leu  Tyr Gln Leu Gln Val  Pro Leu Leu
    1340                1345                1350

Gly Val  Leu Asp Leu Ser Thr  Asn Val Tyr Ser Asn  Leu Tyr Asn
    1355                1360                1365
```

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Trp Ser  Ala Ser Tyr Ser Gly  Gly Asn Thr Ser Thr  Asp His Phe
    1370                 1375                 1380

Ser Leu  Arg Ala Arg Tyr His  Met Lys Ala Asp Ser  Val Val Asp
    1385                 1390                 1395

Leu Leu  Ser Tyr Asn Val Gln  Gly Ser Gly Glu Thr  Thr Tyr Asp
    1400                 1405                 1410

His Lys  Asn Thr Phe Thr Leu  Ser Cys Asp Gly Ser  Leu Arg His
    1415                 1420                 1425

Lys Phe  Leu Asp Ser Asn Ile  Lys Phe Ser His Val  Glu Lys Leu
    1430                 1435                 1440

Gly Asn  Asn Pro Val Ser Lys  Gly Leu Leu Ile Phe  Asp Ala Ser
    1445                 1450                 1455

Ser Ser  Trp Gly Pro Gln Met  Ser Ala Ser Val His  Leu Asp  Ser
    1460                 1465                 1470

Lys Lys  Lys Gln His Leu Phe  Val Lys Glu Val Lys  Ile Asp Gly
    1475                 1480                 1485

Gln Phe  Arg Val Ser Ser Phe  Tyr Ala Lys Gly Thr  Tyr  Gly Leu
    1490                 1495                 1500

Ser Cys  Gln Arg Asp Pro Asn  Thr Gly Arg Leu Asn  Gly Glu Ser
    1505                 1510                 1515

Asn Leu  Arg Phe Asn Ser Ser  Tyr Leu Gln Gly Thr  Asn Gln Ile
    1520                 1525                 1530

Thr Gly  Arg Tyr Glu Asp Gly  Thr Leu Ser Leu Thr  Ser Thr Ser
    1535                 1540                 1545

Asp Leu  Gln Ser Gly Ile Ile  Lys Asn Thr Ala  Ser  Leu Lys Tyr
    1550                 1555                 1560

Glu Asn  Tyr Glu Leu Thr Leu  Lys Ser Asp Thr Asn  Gly Lys Tyr
    1565                 1570                 1575

Lys Asn  Phe Ala Thr Ser Asn  Lys Met Asp  Met Thr  Phe Ser Lys
    1580                 1585                 1590
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Gln Asn Ala Leu Leu Arg Ser Glu Tyr Gln Ala Asp Tyr Glu Ser
1595 1600 1605

Leu Arg Phe Phe Ser Leu Leu Ser Gly Ser Leu Asn Ser His Gly
1610 1615 1620

Leu Glu Leu Asn Ala Asp Ile Leu Gly Thr Asp Lys Ile Asn Ser
1625 1630 1635

Gly Ala His Lys Ala Thr Leu Arg Ile Gly Gln Asp Gly Ile Ser
1640 1645 1650

Thr Ser Ala Thr Thr Asn Leu Lys Cys Ser Leu Leu Val Leu Glu
1655 1660 1665

Asn Glu Leu Asn Ala Glu Leu Gly Leu Ser Gly Ala Ser Met Lys
1670 1675 1680

Leu Thr Thr Asn Gly Arg Phe Arg Glu His Asn Ala Lys Phe Ser
1685 1690 1695

Leu Asp Gly Lys Ala Ala Leu Thr Glu Leu Ser Leu Gly Ser Ala
1700 1705 1710

Tyr Gln Ala Met Ile Leu Gly Val Asp Ser Lys Asn Ile Phe Asn
1715 1720 1725

Phe Lys Val Ser Gln Glu Gly Leu Lys Leu Ser Asn Asp Met Met
1730 1735 1740

Gly Ser Tyr Ala Glu Met Lys Phe Asp His Thr Asn Ser Leu Asn
1745 1750 1755

Ile Ala Gly Leu Ser Leu Asp Phe Ser Ser Lys Leu Asp Asn Ile
1760 1765 1770

Tyr Ser Ser Asp Lys Phe Tyr Lys Gln Thr Val Asn Leu Gln Leu
1775 1780 1785

Gln Pro Tyr Ser Leu Val Thr Thr Leu Asn Ser Asp Leu Lys Tyr
1790 1795 1800

Asn Ala Leu Asp Leu Thr Asn Asn Gly Lys Leu Arg Leu Glu Pro
1805 1810 1815

Leu Lys Leu His Val Ala Gly Asn Leu Lys Gly Ala Tyr Gln Asn
1820 1825 1830

Asn Glu Ile Lys His Ile Tyr Ala Ile Ser Ser Ala Ala Leu Ser
1835 1840 1845

Ala Ser Tyr Lys Ala Asp Thr Val Ala Lys Val Gln Gly Val Glu
1850 1855 1860

Phe Ser His Arg Leu Asn Thr Asp Ile Ala Gly Leu Ala Ser Ala
1865 1870 1875

Ile Asp Met Ser Thr Asn Tyr Asn Ser Asp Ser Leu His Phe Ser
1880 1885 1890

Asn Val Phe Arg Ser Val Met Ala Pro Phe Thr Met Thr Ile Asp
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Ala His Thr Asn Gly Asn Gly Lys Leu Ala Leu Trp Gly Glu His
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Thr Gly Gln Leu Tyr Ser Lys Phe Leu Leu Lys Ala Glu Pro Leu
1925 1930 1935

Ala Phe Thr Phe Ser His Asp Tyr Lys Gly Ser Thr Ser His His
1940 1945 1950

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1955 1960 1965

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1970 1975 1980

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1985 1990 1995

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2000 2005 2010

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2015 2020 20 25

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Asp Ala Val Glu Lys Pro Gln Glu Phe Thr Ile Val Ala Phe Val

2045 2050 2055

Lys Tyr Asp Lys Asn Gln Asp Val His Ser Ile Asn Leu Pro Phe
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Phe Glu Thr Leu Gln Glu Tyr Phe Glu Arg Asn Arg Gln Thr Ile
2075 2080 2085

Ile Val Val Val Glu Asn Val Gln Arg Asn Leu Lys His Ile Asn
2090 2095 2100

Ile Asp Gln Phe Val Arg Lys Tyr Arg Ala Ala Leu Gly Lys Leu
2105 2110 2115

Pro Gln Gln Ala Asn Asp Tyr Leu Asn Ser Phe Asn Trp Glu Arg
2120 2125 2130

Gln Val Ser His Ala Lys Glu Lys Leu Thr Ala Leu Thr Lys Lys
2135 2140 2145

Tyr Arg Ile Thr Glu Asn Asp Ile Gln Ile Ala Leu Asp Asp Ala
2150 2155 2160

Lys Ile Asn Phe Asn Glu Lys Leu Ser Gln Leu Gln Thr Tyr Met
2165 217 0 2175

Ile Gln Phe Asp Gln Tyr Ile Lys Asp Ser Tyr Asp Leu His Asp
2180 2185 2190

Leu Lys Ile Ala Ile Ala Asn Ile Ile Asp Glu Ile Ile Glu Lys
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Leu Lys Ser Leu Asp Glu His Tyr His Ile Arg Val Asn Leu Val
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Lys Thr Ile His Asp Leu His Leu Phe Ile Glu Asn Ile Asp Phe
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Asn Lys Ser Gly Ser Ser Thr Ala Ser Trp Ile Gln Asn Val Asp
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Thr Lys Tyr Gln Ile Arg Ile Gln Ile Gln Glu Lys Leu Gln Gln
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Leu Lys Arg His Ile Gln Asn Ile Asp Ile Gln His Leu Ala Gly
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    2300                 2305                 2310

Leu Glu  His Val Lys His Phe  Val Ile Asn Leu Ile  Gly Asp Phe
    2315                 2320                 2325

Glu Val  Ala Glu Lys Ile Asn  Ala Phe Arg Ala Lys  Val His Glu
    2330                 2335                 2340

Leu Ile  Glu Arg Tyr Glu Val  Asp Gln Gln Ile Gln  Val Leu Met
    2345                 2350                 2355

Asp Lys  Leu Val Glu Leu Thr  His Gln Tyr Lys Leu  Lys Glu Thr
    2360                 2365                 2370

Ile Gln  Lys Leu Ser Asn Val  Leu Gln Gln Val Lys  Ile Lys Asp
    2375                 2380                 2385

Tyr Phe  Glu Lys Leu Val Gly  Phe Ile Asp Asp Ala  Val Lys Lys
    2390                 2395                 2400

Leu Asn  Glu Leu Ser Phe Lys  Thr Phe Ile Glu Asp  Val Asn L ys
    2405                 2410                 2415

Phe Leu  Asp Met Leu Ile Lys  Lys Leu Lys Ser Phe  Asp Tyr His
    2420                 2425                 2430

Gln Phe  Val Asp Glu Thr Asn  Asp Lys Ile Arg Glu  Val T hr Gln
    2435                 2440                 2445

Arg Leu  Asn Gly Glu Ile Gln  Ala Leu Glu Leu Pro  Gln Lys Ala
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Glu Ala  Leu Lys Leu Phe Leu  Glu Glu Thr Lys Ala  T hr Val Ala
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Val Tyr  Leu Glu Ser Leu Gln  Asp Thr Lys Ile Thr  Leu Ile Ile
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Val Gly  Gln Val Tyr Ser Thr  Leu Val Thr Tyr Ile  Ser Asp Trp
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Trp Thr  Leu Ala Ala Lys Asn  Leu Thr As p Phe Ala  Glu Gln Tyr
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Ser Ile  Gln Asp Trp Ala Lys  Arg Met Lys Ala Leu  Val Glu Gln
    2570                 2575                 2580

Gly Phe  Thr Val Pro Glu Ile  Lys Th r Ile Leu Gly  Thr Met Pro
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Ala Phe  Glu Val Ser Leu Gln  Ala Leu Gln Lys Ala  Thr Phe Gln
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Thr Pro  Asp Phe Ile Val Pro  Le u Thr Asp Leu Arg  Ile Pro Ser
    2615                 2620                 2625

Val Gln  Ile Asn Phe Lys Asp  Leu Lys Asn Ile Lys  Ile Pro Ser
    2630                 2635                 2640

Arg Phe  Ser Thr Pro Glu Phe  Thr Ile Leu Asn Thr  Phe His Ile
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Pro Ser  Phe Thr Ile Asp Phe  Val Glu Met Lys Val  Lys Ile Ile
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Arg Thr  Ile Asp Gln Met Gln  Asn Ser Glu Leu Gln  Trp Pro Val
    2675                 2680                 2685

Pro Asp  Ile Tyr Leu Arg Asp  Leu Lys Val Glu Asp  Ile Pro Leu
    2690                 2695                 2700

Ala Arg  Ile Thr Leu Pro Asp  Phe Arg Leu Pro Glu  Ile Ala Ile
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Pro Glu  Phe Ile Ile Pro Thr  Leu Asn Leu Asn Asp  Phe Gln Val
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Pro Asp  Leu His Ile Pro Glu  Phe Gln Leu Pro His  Ile Ser His
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Thr Ile  Glu Val Pro Thr Phe  Gly Lys Leu Tyr Ser  Ile Leu Lys
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Ile Gln  Ser Pro Leu Phe Thr  Leu Asp Ala Asn Ala  Asp Ile Gly
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Asn Gly  Thr Thr Ser Ala Asn  Glu Ala Gly Ile Ala  Ala Ser Ile
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Thr Ala  Lys Gly Glu Ser Lys  Leu Glu Val Leu Asn  Phe Asp Phe
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Gln Ala  Asn Ala Gln Leu Ser  Asn Pro Lys Ile Asn  Pro Leu Ala
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Leu Lys  Glu Ser Val Lys Phe  Ser Ser Lys Tyr Leu  Arg Thr Glu
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His Gly  Ser Glu Met Leu Phe  Phe Gly Asn Ala Ile  Glu Gly Lys
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Ser Asn  Thr Val Ala Ser Leu  His Thr Glu Lys Asn  Thr Leu Glu
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Leu Ser  Asn Gly Val Ile Val  Lys Ile Asn Asn Gln  Leu Thr Leu
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Asp Ser  Asn Thr Lys Tyr Phe  His Lys Leu Asn Ile  Pro Lys Leu
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Asp Phe  Ser Ser Gln Ala Asp  Leu Arg Asn Glu Ile  Lys Thr Leu
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Leu Lys  Ala Gly His Ile Ala  Trp Thr Ser Ser Gly  Lys Gly Ser
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Trp Lys  Trp Ala Cys Pro Arg  Phe Ser Asp Glu Gly  Thr His Glu
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Ser Gln  Ile Ser Phe Thr Ile  Glu Gly Pro Leu Thr  Ser Phe Gly
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Leu Ser  Asn Lys Ile Asn Ser  Lys His Leu Arg Val  Asn Gln Asn
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Leu Val Tyr Glu Ser Gly Ser Leu Asn Phe Ser Lys Leu Glu Ile
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Gly Arg His Asp Ala His Leu Asn Gly Lys Val Ile Gly Thr Leu
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Ser Thr Asn Asn Glu Gly Asn Leu Lys Val Arg Phe Pro Leu Arg
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Leu Thr Gly Lys Ile Asp Phe Leu Asn Asn Tyr Ala Leu Phe Leu
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Ser Pro Ser Ala Gln Gln Ala Ser Trp Gln Val Ser Ala Arg Phe
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Asn Gln Tyr Lys Tyr Asn Gln Asn Phe Ser Ala Gly Asn Asn Glu
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Asn Ile Met Glu Ala His Val Gly Ile Asn Gly Glu Ala Asn Leu
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Asp Phe Leu Asn Ile Pro Leu Thr Ile Pro Glu Met Arg Leu Pro
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Tyr Thr Ile Ile Thr Thr Pro Pro Leu Lys Asp Phe Ser Leu Trp
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Glu Lys Thr Gly Leu Lys Glu Phe Leu Lys Thr Thr Lys Gln Ser
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Phe Asp Leu Ser Val Lys Ala Gln Tyr Lys Lys Asn Lys His Arg
3170 3175 3180

His Ser Ile Thr Asn Pro Leu Ala Val Leu Cys Glu Phe Ile Ser
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Gln Ser Ile Lys Ser Phe Asp Arg His Phe Glu Lys Asn Arg Asn
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Asn Ala Leu Asp Phe Val Thr Lys Ser Tyr Asn Glu Thr Lys Ile
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Lys Phe Asp Lys Tyr Lys Ala Glu Lys Ser His Asp Glu Leu Pro
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Arg Thr Phe Gln Ile Pro Gly Tyr Thr Val Pro Val Val Asn Val
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Glu Val Ser Pro Phe Thr Ile Glu Met Ser Ala Phe Gly Tyr Val
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Phe Pro Lys Ala Val Ser Met Pro Ser Phe Ser Ile Leu Gly Ser
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Asp Val Arg Val Pro Ser Tyr Thr Leu Ile Leu Pro Ser Leu Glu
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Leu Pro Val Leu His Val Pro Arg Asn Leu Lys Leu Ser Leu Pro
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His Phe Lys Glu Leu Cys Thr Ile Ser His Ile Phe Ile Pro Ala
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Met Gly Asn Ile Thr Tyr Asp Phe Ser Phe Lys Ser Ser Val Ile
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Thr Leu Asn Thr Asn Ala Glu Leu Phe Asn Gln Ser Asp Ile Val
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Ala His Leu Leu Ser Ser Ser Ser Ser Val Ile Asp Ala Leu Gln
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Tyr Lys Leu Glu Gly Thr Thr Arg Leu Thr Arg Lys Arg Gly Leu
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Lys Leu Ala Thr Ala Leu Ser Leu Ser Asn Lys Phe Val Glu Gly
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Ser His Asn Ser Thr Val Ser Leu Thr Thr Lys Asn Met Glu Val
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Ser Val Ala Lys Thr Thr Lys Ala Glu Ile Pro Ile Leu Arg Met
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Asn Phe Lys Gln Glu Leu Asn Gly Asn Thr Lys Ser Lys Pro Thr
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Val Ser Ser Ser Met Glu Phe Lys Tyr Asp Phe Asn Ser Ser Met
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Leu Tyr Ser Thr Ala Lys Gly Ala Val Asp His Lys Leu Ser Leu
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Glu Ser Leu Thr Ser Tyr Phe Ser Ile Glu Ser Ser Thr Lys Gly
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Leu Glu Leu Ser Pro Trp Gln Met Ser Ala Leu Val Gln Val His
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Ala Ser Gln Pro Ser Ser Phe His Asp Phe Pro Asp Leu Gly Gln
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Glu Val Ala Leu Asn Ala Asn Thr Lys Asn Gln Lys Ile Arg Trp
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Lys Asn Glu Val Arg Ile His Ser Gly Ser Phe Gln Ser Gln Val
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Glu Leu Ser Asn Asp Gln Glu Lys Ala His Leu Asp Ile Ala Gly
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Ser Leu Glu Gly His Leu Arg Phe Leu Lys Asn Ile Ile Leu Pro
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Val Tyr Asp Lys Ser Leu Trp Asp Phe Leu Lys Leu Asp Val Thr
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Thr Ser Ile Gly Arg Arg Gln His Leu Arg Val Ser Thr Ala Phe
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Val Tyr Thr Lys Asn Pro Asn Gly Tyr Ser Phe Ser Ile Pro Val
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Asp Leu Asn Ser Val Leu Val Met Pro Thr Phe His Val Pro Phe
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Thr Asp Leu Gln Val Pro Ser Cys Lys Leu Asp Phe Arg Glu Ile
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Gln Ile Tyr Lys Lys Leu Arg Thr Ser Ser Phe Ala Leu Asn Leu
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Pro Thr Leu Pro Glu Val Lys Phe Pro Glu Val Asp Val Leu Thr
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Lys Tyr Ser Gln Pro Glu Asp Ser Leu Ile Pro Phe Phe Glu Ile
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Ala Asn Lys Ile Ala Asp Phe Glu Leu Pro Thr Ile Ile Val Pro
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Glu Gln Thr Ile Glu Ile Pro Ser Ile Lys Phe Ser Val Pro Ala
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Gly Ile Val Ile Pro Ser Phe Gln Ala Leu Thr Ala Arg Phe Glu

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Val Asp Ser Pro Val Tyr Asn Ala Thr Trp Ser Ala Ser Leu Lys
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Asn Lys Ala Asp Tyr Val Glu Thr Val Leu Asp Ser Thr Cys Ser
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Ser Thr Val Gln Phe Leu Glu Tyr Glu Leu Asn Val Leu Gly Thr
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His Lys Ile Glu Asp Gly Thr Leu Ala Ser Lys Thr Lys Gly Thr
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Leu Ala His Arg Asp Phe Ser Ala Glu Tyr Glu Glu Asp Gly Lys
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Glu Gly  Gln Ala Ser Phe Gln  Gly Leu Lys Asp Asn  Val Phe Asp
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Gly Leu  Val Arg Val Thr Gln  Lys Phe His Met Lys  Val Lys His
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Glu Ala  Gln Glu Val Phe Lys  Ala Ile Gln Ser Leu  Lys Thr Thr
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Glu Val  Leu Arg Asn Leu Gln  Asp Leu Leu Gln Phe  Ile Phe Gln
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Leu Ile  Glu Asp Asn Ile Lys  Gln Leu Lys Glu Met  Lys Phe Thr
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Tyr Leu  Ile Asn Tyr Ile Gln  Asp Glu Ile Asn Thr  Ile Phe A sn
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Glu Ala  Ser Gln Glu Leu Gln  Gln Ile His Gln Tyr  Ile Met Ala
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Lys Tyr  Tyr Glu Leu Glu Glu  Lys Ile Val Ser Leu  Ile Lys Asn
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Leu Leu  Val Ala Leu Lys Asp  Phe His Ser Glu Ty r  Ile Val Ser
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Ala Ser  Asn Phe Thr Ser Gln  Leu Ser Ser Gln Val  Glu Gln Phe
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Asp Gly  Lys Gly Lys Glu Lys  Ile Ala Glu Leu Ser  Ala Thr Ala
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Gln Glu  Ile Ile Lys Ser Gln  Ala Ile Al a Thr Lys  Lys Ile Ile
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Ser Asp  Tyr His Gln Gln Phe  Arg Tyr Lys Leu Gln  Asp Phe Ser
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Asp Gln  Leu Ser Asp Tyr Tyr  Glu Ly s Phe Ile Ala  Glu Ser Lys
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Arg Leu  Ile Asp Leu Ser Ile  Gln Asn Tyr His Thr  Phe Leu Ile
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Tyr Ile  Thr Glu Leu Leu Lys  Ly s Leu Gln Ser Thr  Thr Val Met
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Gly Arg Phe Trp Asp Tyr Leu Arg Trp Val Gln Thr Leu Ser Glu Gln
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Val Gln Glu Glu Leu Leu Ser Ser Gln Val Thr Gln Glu Leu Arg Ala
65                  70                  75                  80

Leu Met Asp Glu Thr Met Lys Glu Leu Lys Ala Tyr Lys Ser Glu Leu
                85                  90                  95

Glu Glu Gln Leu Thr Pro Val Ala Glu Glu Thr Arg Ala Arg Leu Ser
            100                 105                 110

Lys Glu Leu Gln Ala Ala Gln Ala Arg Leu Gly Ala Asp Met Glu Asp
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Val Cys Gly Arg Leu Val Gln Tyr Arg Gly Glu Val Gln Ala Met Leu
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Gly Gln Ser Thr Glu Glu Leu Arg Val Arg Leu Ala Ser His Le u Arg
145                 150                 155                 160

Lys Leu Arg Lys Arg Leu Leu Arg Asp Ala Asp Asp Leu Gln Lys Arg
                165                 170                 175

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Ser Ala Ile Arg Glu Arg Leu Gly Pro Leu Val Glu Gln Gly Arg Val
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Arg Ala Ala Thr Val Gly Ser Leu Ala Gly Gl n Pro Leu Gln Glu Arg
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Val Arg Ala Lys Leu Glu Glu Gln Ala Gln Gln Ile Arg Leu Gln Ala
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Glu Ala Phe Gln Ala Arg Leu Lys Ser Trp Phe Glu Pro Leu Val Glu
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/051170

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 G01N33/68 G01N33/92 C12Q1/68 A61K31/00 A61K38/00
A61K39/00
//C07K14/775

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 G01N C12Q A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, CHEM ABS Data, WPI Data, EMBASE, BIOSIS, COMPENDEX

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LINGAPPA VR ET AL: "Translocational pausing and the regulation of membrane protein biogenesis" MEMBRANE PROTEINS: STRUCTURE, FUNCTION AND EXPRESSION CONTROL KYUSHU UNIVERSITY PRESS, 7-1-146, HAKOZAKI, HIGASHI-KU, FUKUOKA 812, JAPAN; S. KARGER AG, P.O. BOX, ALLSCHWILERSTRASSE 10, CH-4009 BASEL, SWITZERLAND, 1997, pages 93-100, XP001183818 & INTERNATIONAL SYMPOSIUM ISSN: 3-8055-6465-1 page 95, paragraph 4 - page 97, paragraph 2; figure 2 ----- -/--	1-28

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
6 October 2004	02/11/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Jenn, T

Form PCT/ISA/210 (second sheet) (January 2004)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	BAUMANN, MARC H. ET AL: "Apolipoprotein E includes a binding site which is recognized by several amyloidogenic polypeptides" BIOCHEMICAL JOURNAL, vol. 349, no. 1, 1 July 2000 (2000-07-01), pages 77-84, XP002262330 cited in the application abstract; figures 2,4 page 78, column 1, paragraph 4	1,3
Y	WO 02/065133 A (SALAMA ABDULGABAR; KIESEWETTER HOLGER (DE)) 22 August 2002 (2002-08-22) cited in the application abstract; claim 1; examples 11-13	1,3
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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 99/15159 A (NOVA MOLECULAR INC) 1 April 1999 (1999-04-01) cited in the application abstract; claims 1,17,19,24,31,32,36	9-14
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A	DIEDRICH, JANE F. ET AL: "Neuropathological changes in scrapie and Alzheimer's disease are associated with increased expression of apolipoprotein E-- and cathepsin D in astrocytes" JOURNAL OF VIROLOGY, vol. 65, no. 9, September 1991 (1991-09), pages 4759-4768, XP000443989 cited in the application abstract page 4759, column 2, line 5 - line 10 page 4764, column 2; figures 3,4	16-18,28
A	CHOE, LEILA H. ET AL: "Apolipoprotein E and other cerebrospinal fluid proteins differentiate ante mortem variant Creutzfeldt-Jakob disease from ante mortem sporadic Creutzfeldt-Jakob disease" ELECTROPHORESIS, vol. 23, no. 14, 14 July 2002 (2002-07-14), pages 2242-2246, XP002262331 cited in the application abstract page 2242, column 1, paragraph 1 - column 2, paragraph 1 page 2244, column 2, paragraph 2	16-18,28

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GOLAZ OLIVIER ET AL: "Phenotyping of apolipoprotein E using immobilized pH gradient gels for one-dimensional and two-dimensional separations" ELECTROPHORESIS, vol. 16, no. 7, 1995, pages 1184-1186, XP009021630 ISSN: 0173-0835 cited in the application abstract	16-18,28
A	LUCASSEN RALF ET AL: "In vitro amplification of protease-resistant prion protein requires free sulfhydryl groups" BIOCHEMISTRY;BIOCHEMISTRY APR 15 2003, vol. 42, no. 14, 15 April 2003 (2003-04-15), pages 4127-4135, XP002262517 cited in the application abstract	1-8
A	ENARI M FLECHSIG E WEISSMANN C: "Scrapie prion protein accumulation by scrapie-infected neuroblastoma cells abrogated by exposure to a prion protein antibody" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, NATIONAL ACADEMY OF SCIENCE. WASHINGTON, US, vol. 98, no. 16, 31 July 2001 (2001-07-31), pages 9295-9299, XP002959455 ISSN: 0027-8424 cited in the application abstract	1-8
P,A	US 2004/018554 A1 (GREEN LARRY R) 29 January 2004 (2004-01-29) abstract; claims 13-15	1-8

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box II.1

Although claims 15 to 18 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Continuation of Box II.2

Claims Nos.: 12,22,26

Present claims 12, 22 and 26 relate to an extremely large number of possible use/method/assay. In fact, the claims contain so many options and variables that a lack of clarity (and conciseness) within the meaning of Article 6 PCT arises to such an extent as to render a meaningful search of the claim impossible. Moreover, there is no SEQ ID provided for the fragments of the sequences disclosed in said claims 12, 22 and 26 (Rule 5.2 PCT), and said fragments are defined by reference to a parameter ("is of a molecular weight selected from 30 and 40 kDa). The use of this parameters in the present context is considered to lead to a lack of clarity within the meaning of Article 6 PCT. It is impossible to compare the parameters the applicant has chosen to employ with what is set out in the prior art. The lack of clarity is such as to render a meaningful complete search impossible.

The applicant's attention is drawn to the fact that claims relating to inventions in respect of which no international search report has been established need not be the subject of an international preliminary examination (Rule 66.1(e) PCT). The applicant is advised that the EPO policy when acting as an International Preliminary Examining Authority is normally not to carry out a preliminary examination on matter which has not been searched. This is the case irrespective of whether or not the claims are amended following receipt of the search report or during any Chapter II procedure. If the application proceeds into the regional phase before the EPO, the applicant is reminded that a search may be carried out during examination before the EPO (see EPO Guideline C-VI, 8.5), should the problems which led to the Article 17(2) declaration be overcome.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2004/051170

Box II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 15-18
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 15 to 18 are directed to a diagnostic method practised on the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [X] Claims Nos.: 12,22,26
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

see FURTHER INFORMATION sheet PCT/ISA/210

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [X] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP2004/051170

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International Application No
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